

**Deacylation of glycopeptide antibiotic A40926
by immobilized *Actinoplanes teichomyceticus* cells**
- In quest of better drugs against MRSA

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**Deacylation of glycopeptide antibiotic A40926
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Thesis

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Deacylation of glycopeptide antibiotic A40926 by immobilized *Actinoplanes teichomyceticus* cells - In quest of better drugs against MRSA

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For Maša and Mix...
... and for myself too

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Chapter 1

Introduction

The discovery of antibiotics as antibacterial agents marks a turning point in the history of human health and literarily revolutionized modern medicine. For the past sixty-eight years, the use of antibiotic drugs has turned once life-taking bacterial infections into treatable diseases. With the commercialization of penicillin in 1941 and the rapidly increasing number of newly isolated microbial metabolites having similar antibacterial properties, a general definition was apparently required. In 1947, after much discussion, Waksman¹ realized that the widespread term ‘antibiotic’ in the sense first implied by himself and his collaborators evoked some philological discussion. He therefore advised amending the earlier definition as follows: “An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum. The selective action of an antibiotic is also manifested against microbial versus host cells. Antibiotics vary greatly in their physical and chemical properties and in their toxicity to animals. Because of these characteristics, some antibiotics have remarkable chemotherapeutic potentialities and can be used for the control of various microbial infections in man and in animals.”

Since the introduction of penicillin pharmaceutical companies have produced more than 160 antibacterial agents to combat the wide variety of bacterial infections². Physicians today depend on antibiotics to treat many illnesses caused by bacteria, from rather simple ear and skin infections to more complicated and serious ones such as pneumonia, tuberculosis, polio, scarlet fever, meningitis, and other life-threatening infections. Moreover, antibiotics have become crucial in treating infections that result from medical procedures such as surgery, chemotherapy and transplantations, procedures that are widespread in modern society.

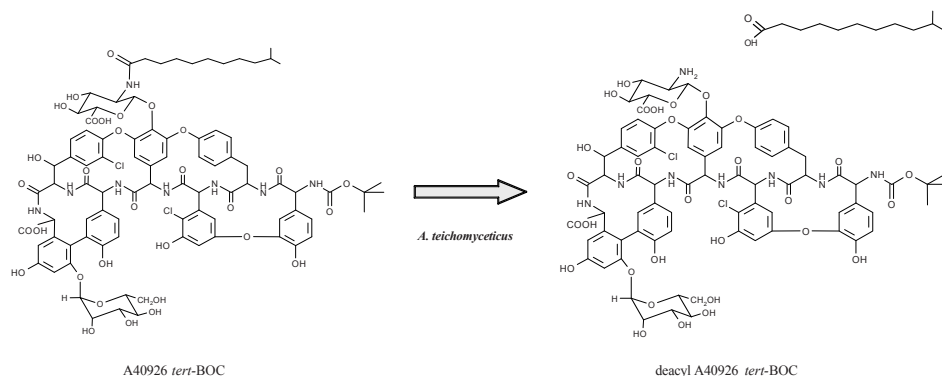


Figure 1 Glycopeptide A40926 and biotransformation to deacyl-A40926 by *Actinoplanes teichomyceticus* cells

Glycopeptides are one of the major classes of antibiotics together with β -lactams (as penicillins and cephalosporins), aminoglycosides, tetracyclines, sulfonamides, macrolides (such as erythromycin) and quinolones. They are a family of compounds of natural origin demonstrating a strong activity against Gram-positive aerobic and anaerobic bacteria³. Typically they are large hydrophilic molecules with molecular masses between approximately 1150 and 2200 Daltons (depending upon the presence or absence of acyl residues and sugars moieties, and on the type of aliphatic and/or aromatic amino acids). A prominent member of the glycopeptide family is A40926 (Fig.1). It demonstrates the high structural complexity of these molecules but also susceptibility to selective deacylation by *Actynoplanes teichomyceticus* cells, a main topic of this thesis research (see last paragraph). The two best known and until today still the only glycopeptide antibiotics commercially available for human use are vancomycin and teicoplanin. Other clinically relevant members of this important class of antibiotics are dalbavancin, oritavancin and telavancin, all semi-synthetic derivatives of natural glycopeptides. Dalbavancin is derived from A40926, oritavancin is obtained from chloroeremomycin, while telavancin is a semi-synthetic derivative of vancomycin. They are all currently in different stages of development. The FDA issued approvable letters for telavancin and dalbavancin in late 2007, and accepted a New Drug Application for oritavancin in early 2008⁴. Glycopeptides are often referred to as drugs of last resort against serious hospital infections caused by multidrug-resistant Gram-positive pathogens, particularly Methicillin-

resistant *Staphylococcus aureus* (MRSA), coagulase-negative staphylococci (CoNS; the coagulase test is used to differentiate *Staphylococcus aureus* (coagulase-positive) from other staphylococci) and enterococci. The great success of vancomycin and teicoplanin are mainly due to efficacy and absence of cross-resistance with other antibiotics (cross-resistance is the tolerance to an antibiotic as a result of exposure to a similarly acting substance), reflecting the unique mode of action of glycopeptides⁵. Unlike β -lactams and all other inhibitors of the biosynthesis of the bacterial cell wall, glycopeptides act by the mechanism of selectively binding to the terminal D-Ala-D-Ala containing precursor of peptidoglycan (a polymer consisting of sugars and amino acids that forms a layer outside the plasma membrane of bacteria, forming the cell wall)⁶.

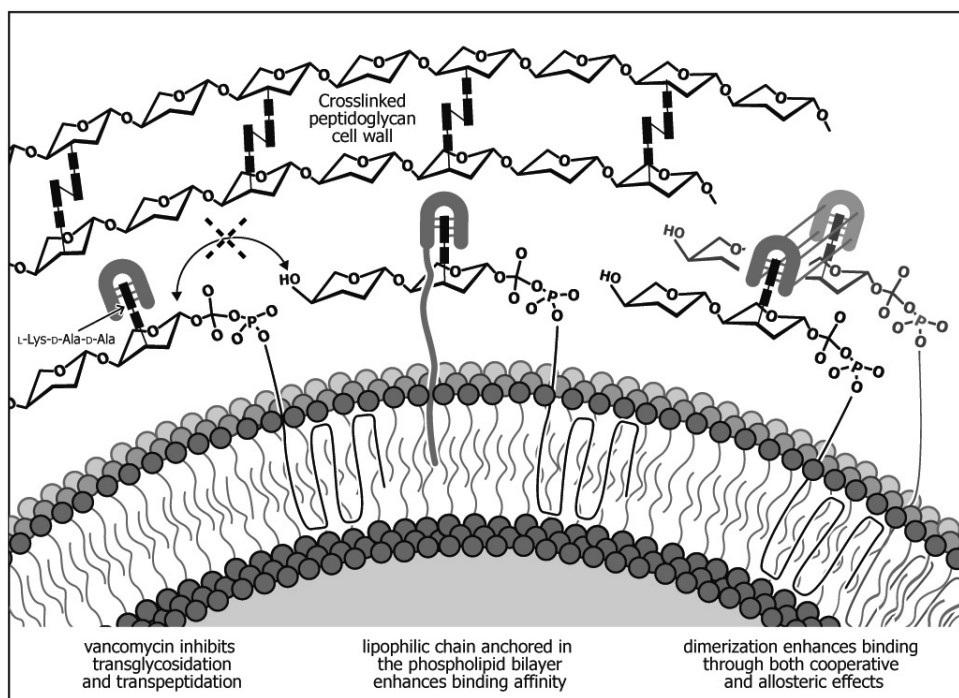


Figure 2 - Mechanisms of action of glycopeptide antibiotics (adapted from Nicolaou et al.⁶). Vancomycin binds to D-Ala-D-Ala, but is unable to bind to the cytoplasmic membrane and is poorly dimerized. By contrast, A40926 with its acyl chain is directly anchored to the membrane and is also a dimer former. These cooperative effects at the target site greatly enhance the activity of A40926.

Basically, transpeptidation and transglycosylation reactions, and consequently cross-linking and polymerization, that are essential for cell wall synthesis, are prevented by the formation of the complex between glycopeptides and bacterial muramyl stem peptides of growing peptidoglycan chains (Figure 2).

Over the years the emergence of bacterial resistance to antibiotics has been observed as a general phenomenon⁷. The extensive use and misuse of antibiotics (such as taking an antibiotic for an inappropriate condition, in particular the use of antibiotics for viral infections, or not taking the entire course of the antibiotic, usually because the patient feels better before the infection is cured) has provided powerful pressure towards selection of resistant microbes. As a consequence, bacteria have mutated or have acquired new genes that allowed them to overcome the action of many antibiotics⁸. Until the mid 1980's, resistance to glycopeptides was rarely observed. However, in 1987 first cases of resistance to vancomycin and teicoplanin were registered in strains of *Enterococcus faecalis* and *Enterococcus faecium*⁹. Resistance is mediated by either of two gene clusters, *vanA* or *vanB*, and it is now widespread among these bacterial species. VanB enterococci are resistant only to vancomycin, while VanA enterococci are highly resistant to both glycopeptides¹⁰. The therapeutic options for severe infections caused by VanA enterococci are still quite limited, resulting in high mortality rates among infected patients. Even though this is considered a serious clinical problem, of a much greater concern is a transfer of the resistance from enterococci to highly virulent MRSA strains and a constant increase in the number of MRSA infections in the hospital¹¹ and community environment¹². Resistance to glycopeptides in MRSA first emerged in Japan in the late 1990's in the form of strains that show reduced susceptibility to vancomycin (named later vancomycin-intermediate *S. aureus* or VISA), or both vancomycin and teicoplanin (named glycopeptides-intermediate *S. aureus* or GISA)¹³. Due to their moderate resistance to glycopeptides, combination therapy with other antibiotics is often needed to treat VISA and especially GISA infections. During the last decade glycopeptides-intermediate *S. aureus* strains have been isolated in numerous countries, particularly from patients having received prolonged therapy. More recently, in different hospital institutions in the United States, 6 new MRSA strains with high levels of resistance to

vancomycin and teicoplanin have been reported ¹⁴. Striking feature is that those cases were *vanA*-mediated infections, suggesting that they have acquired the corresponding set of genes from enterococci. Widespread of such strains in hospital environments is undoubtedly an alarming scenario. Continuous spreading of bacterial resistance in enterococci and staphylococci towards the existing glycopeptide antibiotics became a major concern for the public healthcare authorities ¹⁵. This resulted in an ever-increasing clinical need for new and more potent anti-microbial agents with the challenge to achieve improved activity against multi-resistant *S. aureus* and enterococci ¹⁶.

In a quest of better drugs against MRSA, research efforts carried out by Biosearch Italia (a former Italian company where all of the research presented in this thesis was carried out), resulted in the discovery of A40926, a prominent member of the glycopeptide antibiotic family structurally closely related to teicoplanin¹⁷. A40926 is a commercially very important molecule as it is the precursor for the semi-synthesis of dalbavancin, a novel glycopeptide agent, today a Pfizer Inc. proprietary drug. Dalbavancin is more potent than vancomycin and teicoplanin, and, while sharing many of their positive characteristics it lacks many of their weaknesses. It is highly active against coagulase-negative streptococci and MRSA that are poorly susceptible to teicoplanin. It is also highly active against all streptococci and non-VanA enterococci that are poorly susceptible to vancomycin ¹⁸. In addition, it shows high and prolonged plasma levels (suggesting easier administration and more favorable pharmacokinetics) ¹⁹. Dalbavancin displays strong synergy *in vitro* against VanA enterococci when tested in combination with Ampicillin (a β -lactam antibiotic used extensively to treat bacterial infections)²⁰. It is also active against GISA and shows synergistic activity against GISA isolates in combination with β -lactam antibiotics ²¹. Due to all these positive characteristics, it is considered a second-generation glycopeptide. It combines: 1) the excellent *in vivo* efficacy, 2) very good safety profile, 3) ease of administration, and 4) improved potency against multi-resistant *S. aureus* and enterococci.

Dalbavancin development was a very successful result of a much broader research program at Biosearch Italia to generate improved glycopeptides. An integral part of this research was

the approach to structurally modify A40926. For that the deacylation of A40926 was studied as a starting point to generate a precursor for chemical derivatization. Since attempts of chemical deacylation resulted in extensive side-reactions, a biotransformation route was considered. A large screening program of microorganisms revealed that certain actinomycetes belonging to the *Actinoplanes* genus selectively cleave the fatty acid side chain of the molecule (see Fig.1) to yield the deacylated glycopeptide nucleus²². This derivative can then selectively be reacylated with different acyl chains or functional groups to generate novel compounds with improved antimicrobial activity. Also, deacyl-A40926 was an indispensable element in structure-activity relationships (SAR) studies carried out under the same program. Among the strains selected during the screening program, *Actinoplanes teichomyceticus* was found to be the most efficient in deacylating A40926²². Preliminary characterization of *A. teichomyceticus* deacylase described it as an endoenzyme associated with membrane integrity²³ and the development of a whole cell biotransformation process was proposed.

Aim and thesis outline

A40926 is a glycopeptide antibiotic complex. It is a mixture of several structurally very closely related factors that differ only in the type of the fatty acid moiety linked to the aminoglucuronic acid unit of the molecule. The aim of this thesis research was to develop a lab-scale prototype for continuous deacylation of A40926 by immobilized cells of *Actinoplanes teichomyceticus* and to get insight into the origin of structural differences related to the fatty acid moiety of the molecule.

The first step in our research work was to evaluate the feasibility of deacylation by immobilized cells. To this end we immobilized mycelium of *Actinoplanes teichomyceticus* in calcium-alginate gel beads and confronted their performance to suspended cells in a batch and repeated batch fashion (Chapter 2). We observed that immobilized cells can deacylate A40926 evenly well as suspended cells. Moreover, in repeated batch experiments deacylation was successfully maintained for more than a month, suggesting that a continuous process is also practicable. In this phase of the work we also introduced a method of co-immobilization with soybean meal, as an innovative mode of *in situ* supply of nutrient, and found that it improves deacylation capability of immobilized mycelium.

In order to design any process based on an immobilized biocatalyst, effective diffusion coefficients of the substrate and of the product inside the immobilization matrix are essential. In Chapter 3 we describe determination of effective diffusion coefficients of A40926 and its deacylated derivative. These parameters were necessary for simulation calculations and were subsequently used in modeling of the continuous process in a single airlift bioreactor (Chapter 4) and in a cascade of three airlift bioreactors (Chapter 5).

In addition to the effective diffusion coefficients, kinetic parameters are crucial values for examination of any biocatalytic process. The first part of Chapter 4 is dedicated to the determination of the deacylation kinetic parameters. We describe the deacylation by a Michaelis-Menten kinetic model and determined V_{max} and K_m values that were needed for

further studies. In the second part of Chapter 4 we address the continuous deacylation in a single lab-scale airlift bioreactor (ALR). The performance of a continuously operated ALR is evaluated by the model describing simultaneous diffusion and reaction in the biocatalyst beads. The ALR was operated continuously for 3 weeks with a constant conversion maintained above 60%, indicating good system stability. The experimental results agree well with the model estimates and the applicability of the proposed model for the design of a continuous deacylation process is thus demonstrated.

Because of the very high costs of A40926 (the substrate for deacylation) and the high purity of the final product (deacyl-A40926) needed, a high overall conversion of $\geq 99\%$ is required. By model simulations it was estimated that in a single continuous ALR such a high conversion could be achieved only at very long and impracticable residence times. This called for an alternative and operationally viable solution. Consequently, we examined and simulated other bioreactor operational modes. Considering diffusion and kinetic parameters, demonstrated stability of the system and quite high conversion ($>60\%$) obtained in a single ALR, a possible solution was found in a cascade of airlift bioreactors. In Chapter 5 we describe the deacylation in a series of three perfectly mixed airlift bioreactors. A continuously operated cascade was designed using a model for a series of reactors with immobilized biocatalyst beads obeying Michaelis-Menten kinetics. The cascade system was operated continuously for almost two months and the model estimates for reactor volumes and relative conversions were found to be in good agreement with the experimental results. Under the operational regime implemented, a desired overall conversion of 99% was achieved, demonstrating the feasibility of the continuous deacylation process in a cascade configuration of 3 ALRs.

A40926 is a complex mixture consisting of several structurally related factors. They differ in the structure of the acyl chain (Fig.1), subject of deacylation. In Chapter 6 we describe the origin of structural differences of the single components and demonstrate that the A40926 antibiotic complex composition closely reflects the fatty acid pattern present in the cell membrane of the producing microorganism. This finding is of particular value since it

indicates the possibility of selectively changing the composition of the A40926 complex by altering the distribution of cell fatty acids (through addition of suitable precursors, like amino acids). Moreover, it suggests that new derivatives might be obtained by supplementing the fermentation medium with fatty carbons acids bearing desired structural features. The possibility to selectively direct biosynthesis of the A40926 acyl chain to the desired structure is very attractive as it offers a powerful tool to modulate (and eventually enhance) the binding capacity of A40926 derivatives to the membrane. That could lead to target-oriented production of novel and more active A40926 derivatives.

Chapter 7 places this thesis in a broader context of the long lasting battle of man against multi-resistant bacteria and highlights some of the recent developments in a never-ending quest of better drugs against MRSA.

References

1. Waksman, S.A., 1947. What Is an Antibiotic or an Antibiotic Substance? *Mycologia* **39**, 565-569
2. Service, R.F., 1995. Antibiotics that resist resistance. *Science*, **270**, 724-727.
3. Goldstein, E.J.C., Citron, D.M., Warren, Y.A., Tyrrell, K.L., Merriam, C.V., Fernandez, H.T. 2006. *In Vitro* Activities of Dalbavancin and 12 Other Agents against 329 Aerobic and Anaerobic Gram-Positive Isolates Recovered from Diabetic Foot Infections. *Antimicrobial Agents and Chemotherapy*, Vol. 50, No. 8, p. 2875-2879.
4. Abbanat, D., Morrow, B., Bush, K. 2008. New agents in development for the treatment of bacterial infections. *Current Opinion in Pharmacology* **8** (5), 582-592.
5. Malabarba, A., Ciabatti, R. 2001. Glycopeptide derivatives. *Current Medicinal Chemistry*, **8** , 1759-1773.
6. Nicolaou, K.C., Boddy, C.N.C., Bräse, S., Winssinger, N. 1999. Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew. Chem.-Int. Edit.* **38**, 2096-2152
7. Gold, H.S., Moellering, R.C. 1996. Antimicrobial-drug resistance. *Drug Therapy* **35** (19), 1445-1453.
8. Taubes, G. 2008. The bacteria fight back. *Science*, **321**, 356-361
9. Uttley, A.H., Collins, C.H., Naidoo, J., George, R.C. 1988. Vancomycin-resistant enterococci. *Lancet*, **1**, 57-58.
10. Allen, N.E., Nicas, T.I. 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. *FEMS Microbiology Reviews*, **26**, 511-532
11. Kuehnert MJ, Hill HA, Kupronis BA, Tokars JI, Solomon SL, Jernigan DB. 2005. Methicillin-resistant–Staphylococcus aureus Hospitalizations, United States. *Emerg Infect Dis* **11** (6), 868-872.
12. Rybak, M.J., LaPlante, K.L. 2005. Community Associated Methicillin-Resistant Staphylococcus Aureus: A Review. *Pharmacotherapy* **25** (1), 74-85.
13. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., Tenover, F.C. 1997. Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother.* **40**, 135-136.
14. Dawn M. Sievert, D.M., Rudrik, J.T., Patel, J.B., McDonald, C.L., Wilkins, M.J., Hageman, J.C. 2008. Vancomycin resistant Staphylococcus aureus in the United States, 2002–2006. *J Infect Dis*, **46**, 668–74.

15. Willems, R.J.L., Top, J., van Santen, M., Robinson, D.A., Coque, T.M., Baquero, F., Grundmann, H., Bonten, M.J.M. 2005. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. *Emerg Infect Dis* **11** (6), 821-828.
16. Payne, D.J. 2008. Desperately seeking new antibiotics. *Science* **321**, 1644-1645
17. Parenti, F., Cavalleri, B. 1990. Novel glycopeptide antibiotics of the dalbaheptide group. *Drugs of the future*, **15** (1), 57-72.
18. Van Bambeke, F. 2004. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Current Opinion in Pharmacology* **4** (5), 471-478.
19. Woodford, N. 2003. Novel agents for the treatment of resistant Gram-positive infections. *Expert Opin. Investing. Drugs*, **12** (2), 117-137.
20. Jones, R.N., Biedenbach, D.J., Johnson, D.M., Pfaller, M.A. 2001. In vitro evaluation of BI 397, a novel glycopeptide antimicrobial agent. *J Chemother.* **13** (3), 244-54.
21. Biedenbach, D.J., Bell, J.M., Sader, H.S., Turnidge, J.D., Jones, R.N. 2009. Activities of Dalbavancin against a Worldwide Collection of 81,673 Gram-Positive Bacterial Isolates. *Antimicrobial Agents and Chemotherapy*, Vol. 53, No. 3, 1260-1263.
22. Borghi, A., Spreafico, F., Beretta, G., Ferrari, P., Goldstein, B.P., Berti, M., Denaro, M., Selva, E. 1996 Deacylation of the Glycopeptide Antibiotic A40926 by *Actinoplanes teichomyceticus* ATCC 31121. *J of Antibiotics*, **49**, 607-609
23. Carrelli, A., Borghi, A., Carrano, L., Gastaldo, L., and Marinelli, F. 1995. Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells. *Journal of Industrial Microbiology*, **15**, 429-433

Chapter 2

Biotransformation of the lipoglycopeptide antibiotic A40926 with immobilized cells of *Actinoplanes teichomyceticus* – *in situ* supply of nutrients

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Summary

Mycelium of *Actinoplanes teichomyceticus* ATCC 31121 was immobilized or co-immobilized with soybean meal in calcium-alginate beads. Immobilized mycelium was used to deacylate A40926 antibiotic complex in a batch and repeated-batch system and the results were compared with the performance of a suspension culture as well as washed mycelium. The specific deacylation rate of suspended cells was $0.19 \text{ mg g}^{-1} \text{ h}^{-1}$, while that of washed and immobilized mycelium was found to be much higher, i.e. 0.62 and $0.67 \text{ mg g}^{-1} \text{ h}^{-1}$, respectively. Biotransformation with suspended mycelium proved not to be attractive, in a repeated-batch system. After 8 days, i.e. two batch cycles, only 40% of the initial biomass was recovered, with a decrease in specific deacylation rate of 50%. In contrast to that, repeated-batch biotransformation with immobilized mycelium could be successfully maintained for longer than 30 days. During the first 15 days of operation, i.e. three batch cycles, the deacylation rates were rather constant, but gradually decreased after the third consecutive batch cycle. Co-immobilization with soybean meal as an *in situ* nutrient source improves deacylation capability of immobilized mycelium.

Introduction

Biotransformation is increasingly exploited as a useful and often unique reaction step in the semi-synthesis of pharmaceuticals, or for structural modifications of complex natural compounds such as antibiotics ¹. Compound A40926 is a lipoglycopeptide antibiotic with a strong inhibitory activity against Gram-positive bacteria and *Neisseria gonorrhoea* ². It is produced by fermentation of *Actinomadura* ATCC 39727 as a complex of two main and five minor components. Individual components of the complex have an identical glycopeptide core but differ in the acid chains attached to the amino group of the glucuronic moiety ³. *Actinoplanes teichomyceticus* ATCC 31121 can deacylate A40926 as well as tert-butoxycarbonyl (*tert*-BOC) A40926, yielding the glycopeptide nucleus, which can be then synthetically reacylated to form new analogs (*tert*-BOC A40926 is a protected derivative of A40926, in which the amino group at C₁₅ is blocked to prevent formation of diacyl analogs during reacylation) ⁴. *A. teichomyceticus* is a filamentous microorganism belonging to a rare genus of actinomycetes, previously discovered as the producer of teicoplanin antibiotic.

In a previous study cells in suspension were used to deacylate A40926 *tert*-BOC ⁵. A batch process was developed and scaled up to 300 L, giving an average deacylation yield of 93 %. With the objective of using the biomass more efficiently and repeatedly, and envisaging the development of a continuous deacylation process, cell immobilization was considered. There are many possible advantages of immobilized cell systems, including re-use of cells, facilitated recovery of products, and continuous operation with non-growing cells. It is also easily possible to apply different media, for instance first one optimal for cell growth and subsequently one optimal for biotransformation. In this paper deacylation of A40926 *tert*-BOC with mycelium of *Actinoplanes teichomyceticus* immobilized in calcium-alginate beads is described.

Materials and Methods

Chemicals

A40926 *tert*-BOC and deacyl A40926 *tert*-BOC were prepared using di-*tert*-butyldicarbonate (Janssen, Geel, Belgium) according to general procedures ⁶. Analytical reference substances of A40926 *tert*-BOC and deacyl A40926 *tert*-BOC were prepared in our laboratories and contained 867 mg/g and 894 mg/g of pure antibiotic respectively. The structure and the identity of the antibiotic complex have been determined using a combination of ¹H n.m.r. spectroscopy, fast atom bombardment mass spectrometry (FAB-MS), chemical degradation and gas-chromatography mass spectrometry (GC-MS)³. Acetonitrile (Carlo Erba, Milan, Italy) was of HPLC grade, and all other reagents were of analytical grade unless otherwise indicated.

Organism and Culture Conditions

A. teichomyceticus ATCC 31121 was maintained in a frozen vegetative state in a cryovials at -80 °C. These stock cultures (working cell bank) were used as inocula (2.5% v/v) for 100 ml preculture medium S/Bis (in g/L: glucose 10, bacto peptone 4, bacto yeast extract 4, MgSO₄ x 7H₂O 0.5, K₂HPO₄ 4) in a 500 ml baffled Erlenmeyer flask. Preculture flasks were incubated for 72 h at 28 °C on a rotary shaker at 200 rpm. All experiments were carried out in baffled shake flasks with a foam stopper, with a liquid-to-air volume ratio of 1:5, and at agitation rate of 200 rpm. Under these conditions Oxygen Transfer Rate was estimated to be around 20 mmol L⁻¹ h⁻¹ that was sufficiently high to avoid possible oxygen depletion.

Method of immobilization

A. teichomyceticus cells were immobilized in calcium alginate by a procedure similar to that previously described ⁷. Three ml of preculture mycelium suspension were mixed with 57 ml of sterile 2.1 % sodium alginate (commercially available from Fluka Chemie AG; Fluka cat.no. 71238) at room temperature. Prior to immobilization sodium alginate was

sterilized by autoclaving for 15 minutes at 120°C (this procedure is routinely used in our laboratories and at Wageningen Agricultural University for sterilizing polysaccharides gels and was proven not to affect significantly the stability of the gel beads). In the case of co-immobilization with soybean meal, 0.6 g of soybean meal was mixed with 57 ml of 2.1 % sodium alginate prior to sterilization. The mixture was extruded drop wise through the inner tube of an autoclaved steel dual nozzle (internal diameter 0.8 mm) into a sterile 0.2 M CaCl_2 solution (large excess). The size of the drops was controlled by a coaxial, sterile, water-saturated airflow in the outer tube. The resulting beads were stirred in the CaCl_2 solution at room temperature for 2 h. After that beads were drained on an autoclaved steel sieve, washed with sterile water and immediately used for the pertinent experiments. All work was carried out in a laminar-air-flow cabinet. The diameter of the beads was measured using a Leitz Wetzlar stereoscope with micrometer ocular (10X magnification). The average bead diameter ($d_{\text{mean}} \pm \text{SD}$) determined from a sample of 60 beads was 1.98 ± 0.09 mm.

Biotransformation with suspended cells

500 ml baffled flasks containing 100 ml of AUR/M medium (in g/L: maltose 20, dextrin 10, meat extract 4, yeast extract 2, peptone 4, soybean meal 15, CaCO_3 2) were inoculated with 4 ml of preculture and incubated for 72 h at 28 °C on a rotary shaker at 200 rpm. After 72 hours of growth, an amount of A40926 *tert*-BOC sodium salt, previously dissolved in sterile water at a concentration of 25 g/L, was added to the cultures, giving a final concentration of 0.4 g/L. Samples were collected every 24 hours to monitor the biotransformation reaction by HPLC analysis.

Biotransformation with washed mycelium

Whole culture or aliquots of 20 ml of a 72 h old AUR/M culture were washed twice with 20 mM Tris-HCl buffer (pH 7.0) and resuspended in 100 ml Tris-HCl buffer in a 500 ml baffled flask. Substrate was added at a final concentration of 0.4 g/L and biotransformation followed daily by HPLC analysis. In a repeated-batch experiment a whole culture (100 ml)

was used and mycelium treated as described above.

Biotransformation with immobilized mycelium

Aliquots of 20 ml of the immobilized beads were incubated in 100 ml of AUR/M medium for 72 h at 28 °C on a rotary shaker at 200 rpm. After 72 hours of growth, beads were drained on an autoclaved steel sieve, washed with sterile water, and transferred to 500 ml baffled flasks containing sterile 20 mM Tris-HCl buffer (pH 7.0) with 0.4 g/L A40926 *tert*-BOC (total volume of 100 ml). Biotransformation was monitored daily by HPLC. For repeated-batch deacylation the beads were, after each cycle, drained on a sieve, washed with sterile water, and transferred to a fresh buffer with 0.4 g/L A40926 *tert*-BOC. End volume of Ca-alginate beads plus liquid was always 100 ml.

Sampling and experimental procedure

Suspended cells and washed mycelium experiments

Each sample point represents one shake flask (e.g. in Fig. 1 eight sample points represent eight parallel shake flasks, each one used for one single point in time). From each shake flask duplicate samples were drawn, and flask discarded. Two such experiments were carried out at different times using the same working cell bank, namely Working Cell Bank I. Values and error bars given in figures are average values $\pm$ SD.

Repeated batch experiments with immobilized cells

Two sets of experiments were carried out using two working cell banks, I and II respectively (see Fig.3a and b). Working Cell Bank I corresponds to a working cell bank used in the experiments with suspended cells and washed mycelium. Working Cell Bank II is prepared at a different time but from the same master cell bank. For each experiment two shake flasks were prepared using beads from the same batch of immobilization. At a sample point a single sample from each of two flasks was drawn. Values and error bars given in figures are average values $\pm$ SD.

Analyses

Biomass measurements

For the estimation of biomass dry weight in suspension, 10 ml samples of culture were vacuum filtered through a pre-dried, weighed Whatman No 1 filter paper, washed on the filter with 20 ml distilled water, and dried at 90 °C to constant weight. All measurements were done in duplicate. For the determination of mycelia dry weight immobilized in alginate beads, 50 beads were dissolved in 2% sodium-hexametaphosphate solution. The suspension was then filtered through a pre-dried, weighed Whatman No 1 filter paper and dried at 90 °C to a constant weight.

HPLC analysis

For a standard solution, a stock solution was prepared by dissolving analytical reference substance in 20 mM TRIS-HCl (pH 7) to obtain a concentration corresponding to 250 mg/ml of pure antibiotic. When stored at 4 °C this solution was found to be stable for four months at least. In analysis the calibration standards were prepared by spiking 20 mM TRIS-HCl (pH 7) buffer with the stock solution to obtain a final concentration of 400 mg/L (external standard).

For sample preparation and chromatographic condition, samples were collected and mixed with an equal amount of acetonitrile. After centrifugation (1200 x g for 10 minutes) the supernatant was injected for HPLC analysis. HPLC separations were done on a 5 µm Hewlett Packard ODS Hypersil column (4.6 x 100 mm) using a linear elution gradient from 5% phase B (0.2% HCOONH₄/CH₃CN 3:7) in phase A (0.2% HCOONH₄/CH₃CN 9:1) to 53% B in A in 35 min. The flow rate was 1.8 ml/min and the injection volume 30 µl. The column effluent was monitored at 254 nm. Linearity of this assay was $r^2 = 0.9986$ in the working concentration range.

A calculation of the concentration of antibiotics was determined by an external standard

procedure. The amount of A40926 *tert*-BOC and deacyl A40926 *tert*-BOC present in the sample was calculated as follows:

$$\text{antibiotic concentration} = \frac{A_s * C * V_{std}}{A_{std} * V_s} * D \quad [\text{mg/L}]$$

where A_s is the area of the peak of the antibiotic in the sample to be assayed, C is concentration [mg/L] of antibiotic in the analytical reference standard, A_{std} is the area of the peak of the antibiotic in the external standard, V_s is volume of the sample assayed, V_{std} is volume of the external standard and D is the dilution factor.

Results and Discussion

Biotransformations with suspended cells, and washed and immobilized mycelium

The results of biotransformations with suspended cells, washed mycelium (whole culture and 20% of culture) and immobilized mycelium are given in Table 1. The whole culture of suspended cells transformed 80% of the initial substrate in 9 days, while washed mycelium (whole culture) convert 96% of the initial substrate in the same period (Figure 1).

Table 1. Yields and deacylation rates of *A.teichomyceticus*

	Biomass (g L ⁻¹)	Maximum deacylation rate (mg L ⁻¹ h ⁻¹)	Maximum deacylation yield (%)	Specific deacylation rate (mg g ⁻¹ h ⁻¹)
Suspended cells (whole culture)	20.0 ±0.50	3.8 ±0.50	80 ±2	0.19 ±0.02
Washed mycelium (whole culture)	19.0 ±0.49	5.0 ±0.48	96 ±2	0.26 ±0.03
Washed mycelium (20% of culture)	4.2 ±0.12	2.6 ±0.23	70 ±1	0.62 ±0.06
Immobilized cells	4.0 ±0.15	2.7 ±0.19	92 ±1	0.67 ±0.05

Rates of accumulation of deacyl A40926 were maximal from day 1 to 2 (washed mycelium of whole culture) and from day 2 to 3 (suspended cells) of the experiments. From these data maximum volumetric deacylation rates (±SD) were calculated to be 3.8 ±0.50 and 5.0 ±0.48 mgL⁻¹h⁻¹ for suspended cells and washed mycelium, respectively. Consequently, the specific deacylation rates of suspended cells was 0.19 ±0.02 mgg⁻¹h⁻¹, which is a 30 % lower rate than that of washed mycelium. These findings confirm a previous premise that the fermentation broth contains competitive substrates and/or inhibitors of the deacylating enzyme and that removal favours the bioconversion ⁵.

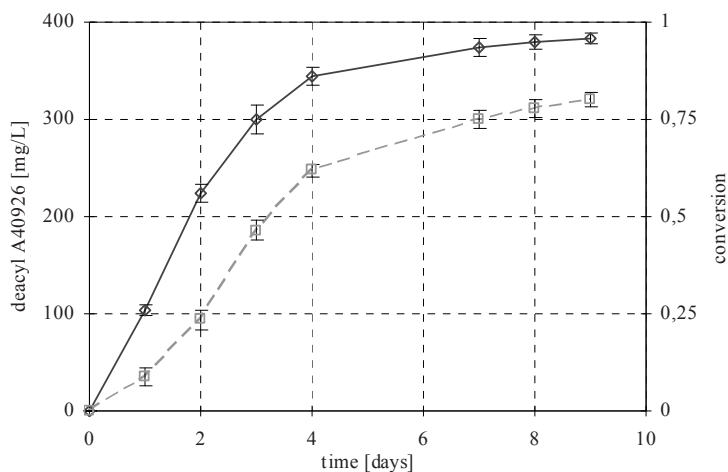


Figure 1 Biotransformation of A40926 *tert*-BOC with washed mycelium of whole culture - ◇, and suspended cells - □, (error bars represent $\pm$ SD).

With immobilized cells deacylation proceeded initially more slowly (Fig. 2). After one day of incubation with immobilized cells almost no deacyl product was detected while the substrate concentration in the liquid phase decreased as much as 30% (data not shown).

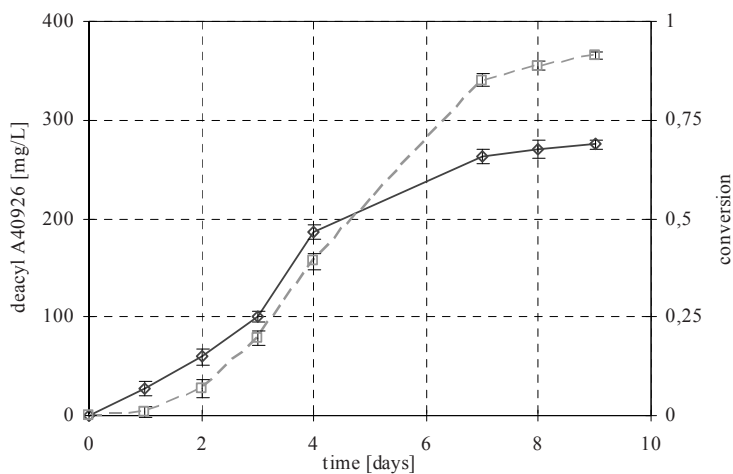


Figure 2 Biotransformation of A40926 *tert*-BOC with immobilized mycelium - □, and washed mycelium of 20 % of culture -◇; (error bars represent $\pm$ SD).

This suggests that during the first hours of incubation substrate diffusion into the gel matrices occurred, but with little concomitant deacylation. As the initially observed decrease in substrate concentration was higher than can be explained by the volume fraction of gel beads, it is probable that the partition coefficient of A40926-*tert* BOC in the gel phase is higher than one. Adsorption phenomena could be involved as well. The characteristics of the *A. teichomyceticus* deacylase are known to be similar to deacylase from other actinomycetes so far described in the literature⁸⁻¹². All those are endoenzymes, with a deacylating activity that is associated with membrane integrity. Thus, it is possible that some sort of temporary adsorption of the antibiotic onto the mycelium is taking place. Further investigations are under way to clarify these points. After two days the reaction proceeded at a constant rate of $2.7 \pm 0.19 \text{ mgL}^{-1}\text{h}^{-1}$, reaching a more than 90 % conversion after 9 days (Fig. 2). The maximum deacylation yield and the specific deacylation rate of immobilized cells were found to be $92 \pm 1\%$ and $0.67 \pm 0.05 \text{ mgg}^{-1}\text{h}^{-1}$, respectively. It is interesting to note that yield achieved with immobilized mycelium was much higher than the one found when the same amount of biomass (20 % of whole culture) was used as a washed mycelium (Table 1 and Fig. 2). These findings are probably explained by improved stability of the metabolic enzymes of immobilized cells. Similar observations have been reported by other authors^{13,14}.

Repeated-batch deacylation with washed and immobilized mycelium

Repeated-batch biotransformations were done with washed mycelium, immobilized mycelium and mycelium co-immobilized with soybean meal using two working cell banks (Figs. 3a and 3b). Mycelia were coimmobilized with 1% (w/v) soybean meal with the idea of providing immobilized cells of *A. teichomyceticus* with the *in situ* nutrient source. Soybean meal is an important component of the growth medium. It is used in the AUR/M medium formulation as a long-term slow-releasing source of nitrogen as well as carbohydrates. Previous experiments with various medium formulations in which soybean meal was substituted with other nutrients (e.g. Soytone) while giving a good mycelium growth failed to deliver a good deacylation capacity of biomass. Thus we postulated that soybean meal might be important for deacylation and that its *in situ* presence in beads

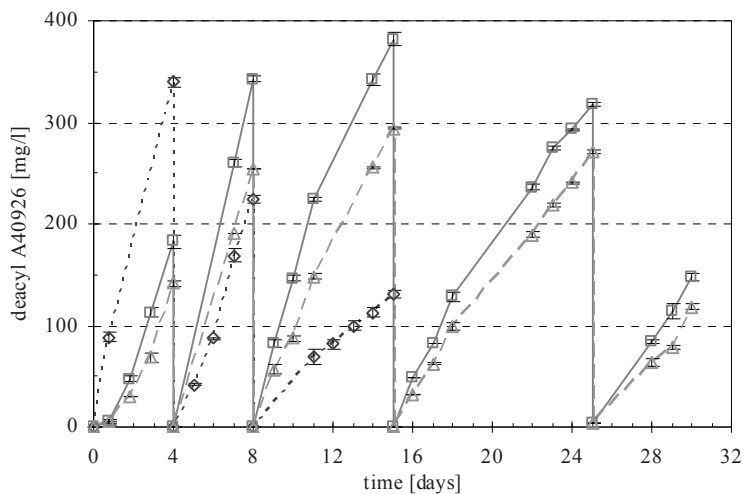
would facilitate this process in a certain way. Data obtained from the experiments with Working Cell Bank I support this premise (Table 2, Fig. 3a).

Table 2. Deacylation rates with washed mycelium and immobilized mycelium in repeated-batch process (data corresponds to Fig. 3-a)

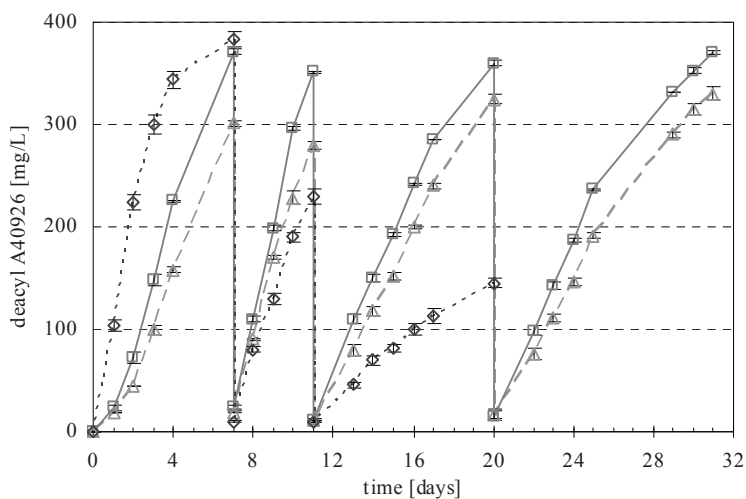
Batch cycles	Maximum volumetric deacylation rate $\pm$ SD ($\text{mg L}^{-1} \text{h}^{-1}$)		
	washed mycelium	immobilized mycelium	mycelium co-immobilized with soybean meal
1	4.4 ± 0.23	2.3 ± 0.08	2.8 ± 0.15
2	2.3 ± 0.17	2.6 ± 0.07	3.6 ± 0.07
3	0.8 ± 0.09	2.0 ± 0.09	3.1 ± 0.09
4	-	1.4 ± 0.05	1.8 ± 0.11
5	-	0.9 ± 0.06	1.2 ± 0.04

When the experiments were repeated with a new batch of the working cell bank, namely Working Cell Bank II (Fig. 3b), the same trends are observed confirming previous findings. At the end of each batch cycle, more deacylated antibiotic was produced with mycelium co-immobilized with soybean meal than with mycelium immobilized without soybean meal.

With washed mycelium the repeated-batch process proved not to be attractive. Volumetric deacylation rates decreased substantially with each new batch (Table 2 and Fig. 3). This is primarily due to a low biomass recovery after each batch cycle. Only 40% of the initial biomass was recovered after two batch cycles (Fig. 4). Such a low biomass recovery resulted in a five times lower volumetric deacylation rate in the third batch as compared to the first one (Table 2). Nevertheless, the specific deacylation rate was constant during the first two cycles, 0.27 and $0.25 \text{ mgg}^{-1}\text{h}^{-1}$, respectively. However, it decreased to about 50% in the third batch, suggesting major loss of catalytic activity. This could be explained by leakage of enzymes due to physical damage of mycelia. Repeated washing/centrifugation steps apparently pose high shear stress on cells, which in turn disturbs cell integrity.



(a)



(b)

Figure 3 Repeated-batch biotransformation of A40926 *tert*-BOC with washed mycelium of whole culture ($\diamond$), immobilized mycelia (Δ), and mycelia co-immobilized with soybean meal ($\square$). Error bars represent $\pm$ SD. Working Cell Bank I (a), Working Cell Bank II (b).

In contrast to repeated-batch deacylation with washed mycelia, biotransformation with immobilized mycelia proved feasible and was successfully maintained for 30 days, during

five batch cycles (Fig.3). The volumetric deacylation rate was rather constant during the first three consecutive batches (Table 2).

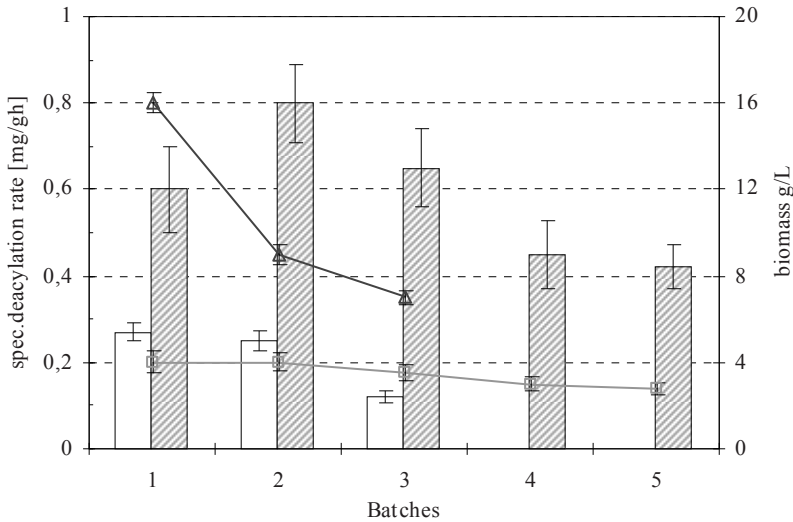


Figure 4 Repeated-batch biotransformation with washed mycelium of whole culture (specific deacylation rate, open histogram; biomass concentration Δ) and mycelium co-immobilized with soybean meal (specific deacylation rate, closed histogram; biomass concentration $\square$). Error bars represent $\pm$ SD. Data related to experiments done with the Working cell Bank I.

However, it decreased thereafter, probably due to loss of biocatalyst. This is explained by observed leakage of biomass from the gel matrix and rupture of some gel beads. The specific deacylation rate also decreased after the third batch cycle (Fig. 4), suggesting a certain degree of enzyme inactivation. Nevertheless, the specific deacylation rate of immobilized mycelium in the fifth batch was still about twice the rate of washed mycelium in the first batch.

Conclusions

Actinoplanes teichomyceticus immobilized in Ca-alginate can be successfully used for deacylation of lipoglycopeptide antibiotic A40926 *tert*-BOC. Co-immobilization with soybean meal improves deacylation capability of immobilized mycelium and should be considered as a novel method for providing *in situ* nutrients. In a repeated-batch biotransformation process immobilized biomass can be effectively used for one month, during five consecutive batch cycles. Experiments are under way to study further deacylation of A40926 *tert*-BOC by immobilized mycelium, and will be subject of our next paper.

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References

1. Sebek, O. Biotransformations. In: *Biotechnology* Vol.6a (Kielisch, K., Rehm, H.J., Reed, G., eds.). Verlag Chemie, Weinheim, 1984, 240-276
2. Goldstein, B.P., E. Selva, L. Gastaldo, M. Berti, R. Pallanza, F. Ripamonti, P. Ferrari, M. Denaro, V. Arioli and G. Cassani. A40926, a new glycopeptide antibiotic with anti- *Neisseria* activity. *Antimicrob. Ag. Chemother.* 1987, **31**, 1961-1966
3. Waltho, J.P., D.H. Williams, E. Selva and P. Ferrari. Structure elucidation of the glycopeptide antibiotic complex A40926. *J. Chem. Soc. Perkin Trans.* 1987, **1**, 2103-2107
4. Borghi A., F. Spreafico, G. Beretta, P. Ferrari, B.P. Goldstein, M. Berti, M. Denaro and E. Selva. Deacylation of the glycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* ATCC 31121. *J. Antibiotics*, 1996, **49**, 607-609
5. Carrelli, A., Borghi, A., Carrano, L., Gastaldo, L., and Marinelli, F. Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells. *Journal of Industrial Microbiology*, 1995, **15**, 429-433
6. Bodanszky M. *Principles of peptide synthesis*. Reversible Blocking of Amino and Carboxyl Groups. Springer-Verlag, Berlin Heidelberg, 1984, 94-102
7. Hulst, A.C., Tramper, J., van 't Riet, K., and Westerbeeck, J.M.M. A new technique for the production of immobilized biocatalyst in large quantities. *Biotechnology and Bioengineering*, 1985, **27**, 870-876
8. Boeck L.B., D.S. Fukuda, B.J. Abbott and M. Debono. Deacylation of A21978C, an acidic lipopeptide antibiotic complex, by *Actinoplanes utahensis*. *J. Antibiotics*, 1988 **41**, 1085-1092
9. Boeck L.B., D.S. Fukuda, B.J. Abbott and M. Debono. Deacylation of echinocandin B by *Actinoplanes utahensis*. *J. Antibiotics*, 1989, **42**, 382-388
10. Chung, S.K., Y.K. Oh, P. Taylor, R. Gerber and I.J. Nisbet. Biosynthetic studies of aridicin antibiotics. II. microbial transformations and glycosylations by protoplasts. *J. Antibiotics*, 1986, **39**, 652-659
11. Kleinschmidt W.J., W.E. Wright, F.W. Kavanagh and W.M. Stark. Penicillin deacylation via *Actinoplanaceae* fermentation. US Patent No. 3, 1962, 150, 059.
12. Singh K. and S. Rakhit. Microbial transformation of leukomycin A. *J. Antibiotics*, 1979, **32**, 78-80
13. Chibata, I., Tosa, T. Use of immobilized cells. *Ann. Rev. Biophys. Bioeng.* 1981, **10**, 197-216
14. Farid, M.A., El Diwany, A.I., El Enshasy, H.A. Production of Oxytetracycline and Rifamycins B and SV with Cells Immobilized on Glass Wool. *Acta Biotechnol.* 1994, **14** (1), 67-74

Chapter 3

Diffusion of (de)acylated antibiotic A40926 in alginate and carrageenan beads with or without cells and/or soybean meal

Summary

Effective diffusion coefficients (D_e) of antibiotic A40926 and its deacylated derivative were determined in Ca-alginate (2% wt/wt) and κ -carrageenan (2.6% wt/wt) gel beads with or without immobilized *Actinoplanes teichomyceticus* cells and/or soybean meal (SBM). The method used was based on transient concentration changes in a well-stirred antibiotic solution in which gel beads, initially free of solute, were suspended. Unsteady-state diffusion in a sphere was applied and D_e determined from the best fit of experimental data. A40926 showed markedly different diffusion characteristics than its deacylated derivative. Diffusivity of deacyl-A40926 in alginate or carrageenan gel beads was six to seven times that of A40926. Large differences in partition coefficients (K_p) were also found. In case of beads without additions, A40926, in contrast to deacyl-A40926, strongly partitioned to the liquid phase. Introduction of SBM and/or mycelium in the gel beads decreased the effective diffusivity of deacyl-A40926, but increased its partitioning to the solid phase. Our findings indicate that a relatively moderate structural change of a lipoglycopeptide molecule could lead to a major change in its diffusion/partition characteristics.

Introduction

Entrapment in polysaccharide gels is one of the most commonly used techniques for immobilization of bacterial cells.¹ This technique is widely accepted because it comprises a simple, mild and non-destructive procedure compatible with viable cells. The most frequently used gels are Ca-alginate and κ -carrageenan, in which case cells are normally immobilized in small spherical beads. In these beads cells can experience a different local environment than when freely suspended in medium. This difference is the result of mass transfer resistance and, in some cases, also due to solute partitioning between solid and liquid phase. Solute partitioning is commonly described by the partition coefficient (Kp), while mass transfer can be described by the external stagnant-film mass-transfer resistance and the internal mass-transfer resistance. Very often, hydrodynamic operative regimes applied in the bioprocesses are such that the external-film mass-transfer resistance is negligible with respect to the internal mass-transfer resistance. Internal mass transfer, i.e. transport of substrates and products inside the gel beads, occurs by diffusion and the effective diffusion coefficient (D_e) is the parameter that mathematically describes the rate of this transport per unit of driving force. Studies of internal mass transfer are thus a prerequisite for a good understanding and proper description of the immobilized biocatalyst system.

The subject of our research is A40926, the proprietary lipoglycopeptide antibiotic of Biosearch Italia. In our laboratories we are currently developing BI397, a semisynthetic derivative of A40926. New and often more specific and more active analogs of this attractive antibiotic can be obtained by synthetic reacylation of its deacylated derivative. To this end A40926 is selectively deacylated by *Actinoplanes teichomyceticus* ATCC 31121 to yield the glycopeptide nucleus (Fig. 1). In our previous work we used immobilized *A.teichomyceticus* for deacylation of A40926.² We demonstrated the feasibility of the immobilized biocatalyst system for this particular biotransformation and examined *in situ* supply of nutrients by co-immobilizing soybean meal in the gel beads. For modeling the

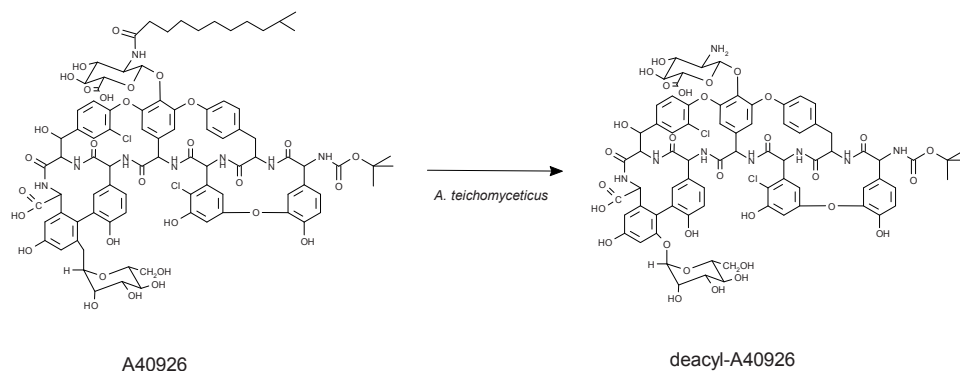


Figure 1 - Biotransformation of A40926 to deacyl-A40926 by *Actinoplanes teichomyceticus* ATCC 31121.

process that will employ immobilized *A. teichomyceticus*, values of the effective diffusion coefficient for the substrate and the product are needed. The main objective of this work was to determine the diffusion characteristics of antibiotic A40926 and its deacylated derivative in Ca-alginate and κ -carrageenan gel beads.

Materials and Methods

Preparation of gel beads

Four different types of beads were used: gel beads without additions, gel beads with co-immobilized soybean meal (SBM; 1% wt/wt), gel beads with entrapped mycelium of *A. teichomyceticus*, and gel beads with 1% SBM and entrapped mycelium. Ca-alginate beads were prepared by the procedure previously described.² κ -carrageenan gel beads were prepared by the same immobilization technique. For that, a 2.6% (wt/wt) solution of κ -carrageenan (Genugel X0828, A/S Kobenhavns Pektinfabrik, Lille Skensved, Denmark), with or without 1% (wt/wt) SBM, was extruded drop wise and beads collected in 0.75 M KCl at 5 °C. A layer of ice-cold n-decane was first brought upon the KCl solution to facilitate formation of spheres, as proposed by Buitelaar *et al.*³ n-Decane is known to be non-toxic to cells because of its high $\log P$ value⁴. The diameter of the beads was measured using a Leitz Wetzlar stereoscope with micrometer ocular (10X magnification). The typical average bead diameter ($d_{\text{mean}} \pm \text{SD}$) determined from a sample of 100 beads was 2.44 ± 0.09 mm. To prepare gel beads with mycelium, aliquots of 20 ml of the beads with 4% inoculum (volume of vegetative culture / volume of gel) were incubated in 100 ml of AUR/M medium for 72 h at 28 °C on a rotary shaker at 200 rpm.² After 72 hours of growth, beads were drained on an autoclaved steel sieve, washed with sterile water, and immediately used for the diffusion experiments. To estimate mycelial dry weight, 50 beads were dissolved in 2% sodium-hexametaphosphate solution. The suspension was then filtered through a pre-dried, weighed Whatman No 1 filter paper and dried at 90 °C to a constant weight. Amount of biomass was calculated to be 20 g per liter of gel.

Diffusion experiments

Diffusion experiments were conducted at 28 °C in a thermostatic room and in a closed vessel to avoid evaporation. All the solutions and the beads used were kept in the same room for at least 2 hours before the experiments were started. To start experiments a known volume of A40926 or deacyl - A40926 solution was placed in a closed cylindrical glass vessel. The solution was agitated on a magnetic stirrer set at 700 rpm to avoid external

mass-transfer resistance. The significance of the external mass-transfer resistance can be evaluated by the Biot number, which is defined as the ratio of the external and internal mass-transfer resistance. Using the procedure described by van't Riet and Tramper⁵ external mass-transfer resistance was estimated. Considering effective diffusivities obtained, Bi was estimated to be > 74 , under experimental conditions, indicating that external mass-transfer resistance could be neglected with respect to the internal mass-transfer resistance.

At time zero a known volume of beads was quickly dropped into the appropriate solution. The transient concentration change of the solute in the liquid phase was followed by HPLC analysis described elsewhere.² The sum of the individual sample volumes taken for the HPLC analysis was in all experiments below 0.1 % of the liquid-phase volume.

Diffusion-coefficient calculations

The theoretical background for the determination of the effective diffusion coefficient is given by Crank.⁶ In this study we used method described by Wijffels *et al.*⁷ with modifications explained below. When spherical beads that are initially free of solute are suspended in a well-stirred solution with an initial solute concentration $C_{s,l,0}$, and provided that the external mass-transfer resistance surrounding the beads is negligible, the transient concentration change of the solute in liquid phase, $C_{s,l}(t)$, is given by:

$$\frac{C_{s,l}(t)}{C_{s,l}(0)} = \frac{\alpha}{1 + \alpha} + \sum_{n=1}^{\infty} \frac{6 \cdot e^{-D_e \cdot q_n^2 \cdot t / R^2}}{9/\alpha + 9 + q_n^2 \cdot \alpha} \quad (1)$$

In Eq. (1), D_e is the effective diffusion coefficient, t is the diffusion time, R is the bead radius, and the q_n 's are positive non-zero roots of the transcendental equation:

$$\tan q_n = \frac{3 \cdot q_n}{3 + \alpha \cdot q_n^2} \quad (2)$$

α is the ratio of the volume of the solution, excluding the volume of the beads, to the volume of the beads divided by the partition coefficient and can be expressed as:

$$\alpha = \frac{3 \cdot V_l}{4 \cdot \pi \cdot R^3 \cdot n_b \cdot K_p} \quad (3)$$

where V_l is the volume of liquid phase, n_b is the total number of beads and K_p is the partition coefficient. The partition coefficient K_p between the gel and the solution is defined as the solute concentration ratio in the beads and in the solution at equilibrium:

$$K_p = \frac{C_{s,b}(\infty)}{C_{s,l}(\infty)} \quad (4)$$

Equation (1) contains D_e and subsequent q_i values as fitting parameters. A curve fitting procedure was applied to numerical solutions to equation (2). As a result, an explicit solution to $q_i = f(\alpha, i)$ was obtained (in which i stands for the i^{th} solution):

$$q_i = 0.064 + 3.107 \cdot i + 0.361/\sqrt{\alpha} \quad (5)$$

Substitution of equation (5) for $i = 1..5$ in equation (1) yielded an explicit expression for $C_{s,l} = f(D_e, \alpha)$ which was then used to fit data on $C_{s,l} = f(t)$, yielding D_e and α values. The α value was then used to calculate the partition coefficient K_p according to equation (3).

Results and Discussion

Figure 2 shows an example of transient deacyl-A40926 concentration in the liquid phase. Antibiotic concentration in the solution decreased, and the equilibrium was reached after about one hour. Equation (1) adequately describes the data. It has been originally developed for a single sphere. Nevertheless, given the small variation in bead size, it can also be applied to a number of spherical beads using the average diameter of the beads.

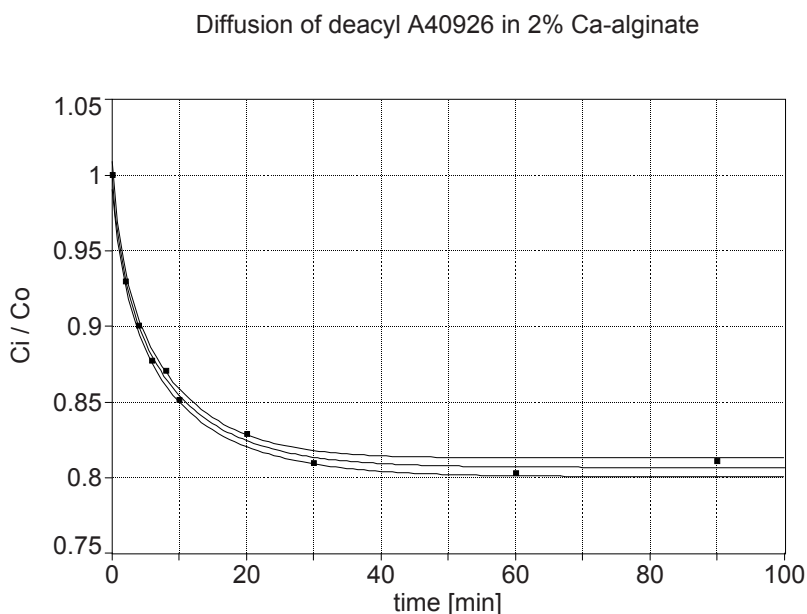


Figure 2 - Diffusion of deacyl-A40926 into alginate beads without additions. Solid line corresponds to the best fit, dashed lines represent 99% confidence region.

The results obtained with the different bead types are summarized in Table 1. In each case experiments were carried out twice and the values for the effective diffusion coefficient and the partition coefficient given are mean values. Diffusion characteristics of deacyl-A40926 in gel beads of alginate and carrageenan without additions are similar considering the small difference in De . However, with alginate the compound tends to partition to the liquid

phase while with carrageenan the opposite was found.

Table 1 - Effective diffusion and partition coefficient of A40926 and deacyl-A40926 in 2% Ca-alginate and 2,6 % κ -carrageenan gel beads

Bead type	A40926		deacyl-A40926	
	$D_e \times 10^{11}$ [m ² s ⁻¹]	K_p [-]	$D_e \times 10^{10}$ [m ² s ⁻¹]	K_p [-]
Alginate	2.86	0.17	2.11	0.87
Alginate +SBM	0.39	16.32	1.41	1.70
Alginate + mycelium	0.97	2.27	1.35	2.32
Alginate +SBM + mycelium	0.53	10.60	1.05	3.38
κ -Carrageenan	3.14	0.29	2.19	1.32
κ -Carrageenan + SBM	0.59	20.01	1.57	1.78

Introduction of SBM and/or mycelium in the gel beads decreases De , but at the same time increases partitioning to the solid phase. The decrease in De was to be expected since soybean meal and/or mycelium inside the gel matrix creates additional internal mass-transfer resistance. Increase in partitioning coefficient towards the beads can be attributed to adsorption. Temporary adsorption of the antibiotic to soybean meal has been observed in previous experiments (unpublished results).

Qualitative behavior of A40926 and deacyl-A40926 are similar considering diffusion in free gel beads (Figure 3). However, there are significant quantitative differences. In the gel beads without additions, De values of A40926 are an order of magnitude lower than those found for the deacyl-A40926. This difference might be attributed to the presence of the long hydrophobic acyl chain on the amino-sugar moiety. Much higher partitioning to the liquid phase was also found. These observations can be explained by the chemical characteristics of A40926. While deacyl-A40926 is present as a free molecule in the aqueous solution, A40926 tends to form pseudo-micelle structures⁸. This is mainly due to

the presence of the long hydrophobic acyl chain that could act as an apolar “tail” of the polar glycopeptide “head”.

Diffusion of A40926 in 2% Ca-alginate

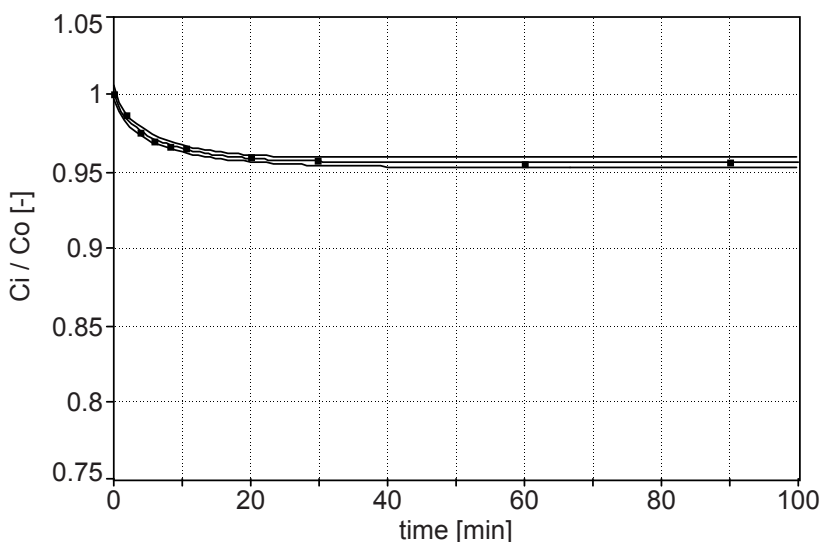


Figure 3 - Diffusion of A40926 into alginate beads without additions. Solid line corresponds to the best fit, dashed lines represent 99% confidence region.

When soybean meal and/or mycelium were immobilized in the gel beads, A40926 strongly partitions to the solid phase. Adsorption of A40926 to the soybean meal has been observed before, as mentioned above. In the case when mycelium was immobilized in the beads, the presence of biocatalyst requires that the reaction of deacylation should be considered as well. However, the reaction is known to have a long lag phase⁹ and the rate is at least an order of magnitude lower than the rate of diffusion in gel beads without additions (unpublished results). Moreover, deacyl-A40926 was not detectable in the liquid phase during the course of the experiments. Still, the presence of biocatalytically active mycelium in the gel beads undoubtedly influences mass transfer characteristics of this system. *A.teichomyceticus* deacylase, enzyme responsible for this biotransformation, is an endoenzyme whose deacylating activity is associated with membrane integrity.¹⁰⁻¹¹

Considering the chemical structure of the substrate, it is likely that A40926 is temporary anchored to the membrane bilayer by its lipophilic acyl chain.⁸ This adhesion event can be seen as a prelude to the catalytical cleavage of the chain itself and is probably another reason why, when mycelium was present in the gel beads, A40926 partitions to the solid phase.

Conclusions

Unsteady-state diffusion in a sphere, as presented here, could be used for the determination of effective diffusion coefficients of antibiotic A40926 in Ca-alginate and κ -carrageenan gel beads. Lipoglycopeptide antibiotic A40926 showed markedly different diffusion/partition characteristics than its deacylated derivative. When soybean meal and/or *A.teichomyceticus* mycelium was present in the gel beads A40926 strongly partitions to the solid phase. For modeling and optimization of the deacylation process both diffusion characteristics of the system and kinetics of the immobilized mycelium are needed. Our current work is directed towards determination of deacylation kinetics and process description by the experimentally determined parameters.

References

1. R.H. Wijffels, R.M. Buitelaar, C. Bucke and J. Tramper (Eds) *Immobilized Cells: Basics and Applications*, Proceedings of an International Symposium organized under auspices of The Working Party on Applied Biocatalysis of the European Federation of Biotechnology, held at Noordwijkerhout, The Netherlands, November 26-29, 1995. Elsevier Science B.V., Amsterdam, 1996.
2. Jovetic, S., Tramper, J. and Marinelli, F. Biotransformation of the lipoglycopeptide antibiotic A40926 with immobilized cells of *Actinoplanes teichomyceticus* - in situ supply of nutrients. *Enzyme Microb. Technol.* 1998, **22**, 117-121
3. Buitelaar R.M., Hulst A.C., Tramper, J. Immobilization of biocatalysts in thermogels using the resonance nozzle for rapid drop formation and an organic solvent for gelling. *Biotechnol. Tech.* 1989, **2**, 109-114
4. Laane C., Boeren S., Vos K., Veeger C. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* 1987, **30**, 81-87
5. van't Riet, K and Tramper, J. *Basic Bioreactor Design*. Marcel Dekker, New York, 1991, pp. 243-224
6. Crank, J. *The Mathematics of Diffusion*, 2nd edition. Oxford University Press, Oxford, 1975, pp. 93-94
7. Wijffels, R.H., Englund, G., Hunik, J.H., Leenen, E.J.T.M., Bakketun, A., Gunther, A., Obon de Castro, J.M., Tramper, J. Effects of diffusion limitation on Immobilized nitrifying microorganisms at low temperatures. *Biotechnol. Bioeng.* 1995, **45**, 1-9
8. Lancini, G. Personal communications
9. Carrelli, A., Borghi, A., Carrano, L., Gastaldo, L., and Marinelli, F. Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells. *Journal of Industrial Microbiology*, 1995, **15**, 429-433
10. Boeck L.B., D.S.Fukuda, B.J.Abbott and M.Debono. Deacylation of A21978C, an acidic lipopeptide antibiotic complex, by *Actinoplanes utahensis*. *J.Antibiotics*, 1988 **41**, 1085-1092
11. Boeck L.B., D.S.Fukuda, B.J.Abbott and M.Debono. Deacylation of echinocandin B by *Actinoplanes utahensis*. *J.Antibiotics*, 1989, **42**, 382-388

Chapter 4

Deacylation of antibiotic A40926 by immobilized *Actinoplanes teichomyceticus* cells in an internal-loop air-lift bioreactor

Abstract

A40926 is a natural glycopeptide antibiotic. It is a precursor of dalbavancin, a semi-synthetic second-generation glycopeptide in clinical development for the treatment of β -lactam-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* infections. Deacylation of A40926, producing an intermediate useful for the synthesis of novel derivatives, can be carried out by the action of *Actinoplanes teichomyceticus* cells. Kinetics parameters for this particular deacylation reaction were obtained by fitting the experimental data to a Michaelis-Menten kinetic model. The feasibility of A40926 deacylation by immobilized *A. teichomyceticus* cells in an air-lift bioreactor was examined. A single continuously operated air-lift reactor (ALR) was evaluated by a model describing diffusion and reaction in the biocatalyst beads. Model estimates were in a good agreement with experimental results. The system was operated continuously for 21 days with overall conversion above 60%.

Introduction

The subject of our research is A40926, the proprietary antibiotic of Biosearch Italia. A40926 is a natural glycopeptide produced by *Nonomuria* sp. ATCC 39727¹. It is a precursor for the semi-synthesis of dalbavancin, a novel second-generation glycopeptide agent belonging to the same class of drugs as vancomycin and teicoplanin. Formerly referred to as BI-397, dalbavancin is designed to be an improved alternative to those antibiotics². In clinical studies to date, it appears to be a potent antibiotic for the treatment of infections by β -lactam-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* strains.

Ever-increasing clinical need and importance of dalbavancin led to a quest for the new analogs of A40926. Screening microorganisms for the ability to transform this antibiotic, *Actinoplanes teichomyceticus* ATCC 31121 was found to selectively cleave the acyl group of A40926 (Fig. 1) and of its protected *tert*-BOC derivative^{3,4}. The deacylated *tert*-BOC A40926 thus obtained represents an attractive intermediate, since deacyl *tert*-BOC A40926 can be chemically reacylated with various side chains to yield new, putatively more specific and active analogs. Moreover, in-depth studies on the influence of aliphatic chain on the antibacterial activity of new analogs and their binding to the target bacterial structure are made possible. In our previous work we used suspended⁴ and immobilized⁵ *A.teichomyceticus* cells for deacylation of A40926 *tert*-BOC. We demonstrated the feasibility of the repeated batch deacylation with immobilized biocatalyst and proposed a continuous process as an alternative to the batch process⁵. For the development and modeling of such a process, kinetic parameters of the biotransformation reaction have to be known. The objective of this work was to determine kinetic parameters and examine A40926 deacylation by immobilized *A. teichomyceticus* in a continuous process.

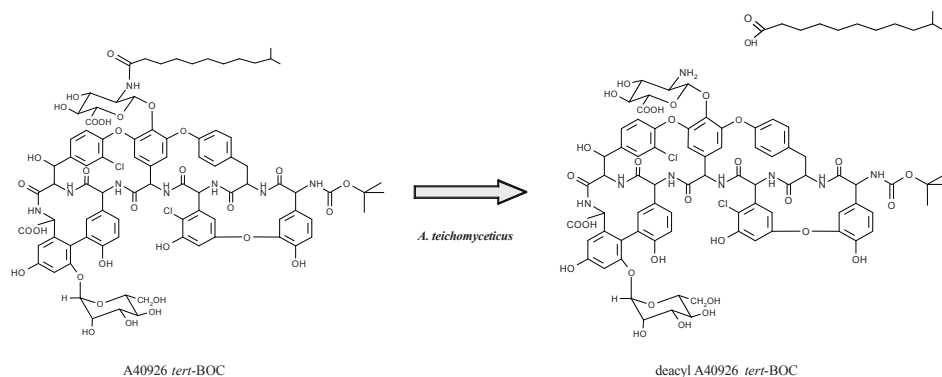


Figure 1 - Biotransformation of A40926 to deacyl-A40926 by *A. teichomyceticus* ATCC 31121.

In the present contribution an internal-loop air-lift reactor (ALR) was used. System was operated continuously for three weeks. Kinetic parameters were obtained by fitting experimental data to the Michaelis-Menten kinetic model while the theoretical development and the calculation procedures of De Gooijer *et al.*⁶ were used as a tool to describe continuous deacylation of A40926 by immobilized biocatalyst. This model described the simultaneous diffusion and conversion of substrate in the biocatalyst beads with or without external-diffusion limitation. Kinetic constants, substrate diffusion coefficient⁷ and other operational parameters were used as input for the model simulations. Calculated estimates were in a good agreement with experimental results.

Materials and Methods

Chemicals

A40926 *tert*-BOC and deacyl A40926 *tert*-BOC (in further text A40926 and deacyl A40926) were prepared as previously described⁵. Acetonitrile (Carlo Erba, Milan, Italy) was of HPLC grade, and all other reagents were of analytical grade unless otherwise indicated.

Organism and Culture Conditions

Actinoplanes teichomyceticus ATCC 31121 was maintained in a frozen vegetative state at -80 °C. The stock cultures were used as inoculums (2.5% v/v) for 100 ml pre-culture medium S/Bis (in g/l: glucose 10, bacto peptone 4, bacto yeast extract 4, MgSO₄ x 7H₂O 0.5, K₂HPO₄ 4) in a 500 ml baffled Erlenmeyer flask. Pre-culture flasks were grown for 72 h at 28 °C on a rotary shaker at 200 rpm, and used as inoculums in subsequent experiments. For the kinetic experiments washed cells of *A. teichomyceticus* were prepared in the following way: 500 ml baffled shake flasks containing 100 ml of AUR/M medium (in g/l: maltose 20, dextrin 10, meat extract 4, yeast extract 2, peptone 4, soybean meal 15, CaCO₃ 2) were inoculated with 4 ml of pre-culture and incubated at 28 °C on a rotary shaker at 200 rpm. After 72 h of growth cultures were washed twice with 20 mM Tris-HCl buffer (pH 7.0) and resuspended in 100 ml Tris-HCl buffer in a 500 ml shake flasks.

Kinetic measurements

For determination of kinetic parameters substrate conversion was measured as a function of time. In two independent experiments ten different initial substrate concentrations were used. A total of 22 shake flasks with washed cells of *A. teichomyceticus* (as described above) were used for kinetic experiments and dry cell weight determinations. At t=0 h, different amounts of A40926 sodium salt, previously dissolved in sterile water at a concentration of 25 g/l, were added to each pair of shake flasks to yield desired initial substrate concentrations. The culture flasks were incubated at 28 °C on a rotary shaker at

200 rpm. Samples were collected at regular time intervals and analyzed by HPLC. All measurements were carried out in duplicate. The initial reaction rates were determined by linear regression. The reaction rates are expressed in mol deacyl-A40926 formed per kg dry cell weight per second.

Kinetic calculations

Previous studies suggest that the deacylation of A40926 by *A. teichomyceticus* cells can be described by Michaelis-Menten kinetics⁴. To determine kinetic parameters a simple Michaelis-Menten expression (Eq. 1) was used,

$$V = V_{\max} * \frac{C_s}{K_m + C_s} \quad (1)$$

where V is the initial reaction rate (mol g⁻¹ h⁻¹), V_{max} is the maximum reaction rate (mol g⁻¹ h⁻¹), C_s is the substrate concentration (mol l⁻¹) and K_m is the Michaelis constant (mol l⁻¹). Parameter estimation was done by fitting Eq. 1 to experimental data pairs of substrate concentrations versus initial reaction rates. TableCurve 2D, (Version 3 for Windows, Jandel Scientific) software was used for data fitting and statistical analysis.

Method of immobilization

Actinoplanes teichomyceticus cells were immobilized in calcium alginate gel by a procedure previously described⁵. All work was carried out in a laminar-air-flow cabinet. The diameter of the beads was measured using a Leitz Wetzlar stereoscope with micrometer ocular (10X magnification). The average bead diameter (d_{mean}±SD) determined from a sample of 120 beads was 2.05 ±0.11 mm. Beads with two gel loads, 53 and 28 kg of dry cell weight per m³ of gel respectively, were obtained and used in two ALR experiments.

Analyses

Concentration of biomass in a suspension and in the immobilized beads was estimated by dry weight measurements described elsewhere⁵. The concentration of A40926 and deacyl-

A40926 was determined by an external standard procedure using analytical reference substances prepared in our laboratories. HPLC analytical method, sample preparation and calculation procedure used were previously described⁵.

ALR experiments

The vessel used in this study was a glass made, internal-loop air-lift reactor (ALR) constructed by the glass workshop of Wageningen University (The Netherlands). The working volume of the reactor was 0.43 l (a cross section of the ALR is given in Fig. 2).

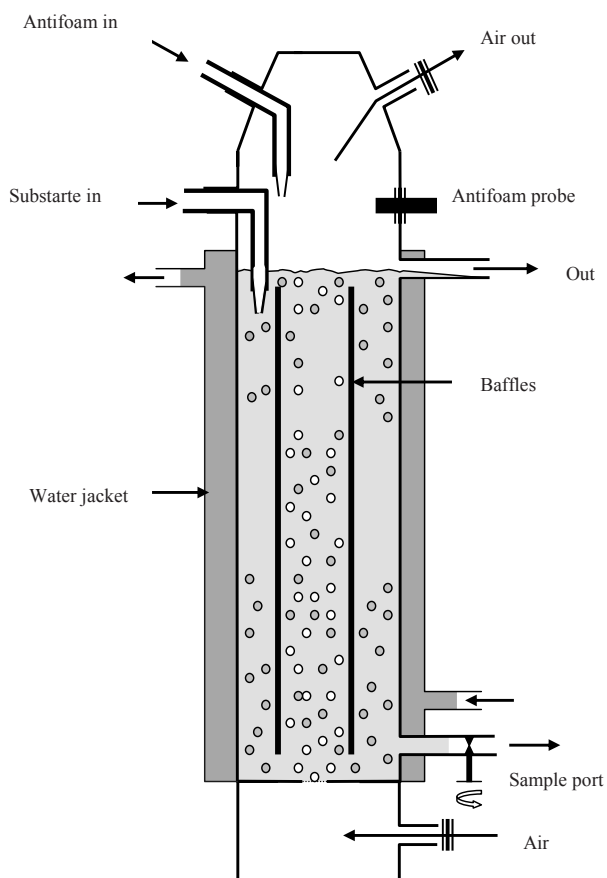


Figure 2 - Cross section of internal loop Air-Lift Reactor.

The temperature in the reactor was kept constant at 28 °C by circulating water in the jacket. A Gilson Minipuls 2 peristaltic pump controlled incoming and outgoing liquid flow. To prevent excessive foaming a Bioengineering antifoam control system was applied with a silicone based antifoam agent. Aeration was executed by sterile air, supplied through the ceramic porous sparger at the bottom of the ALR at a flow rate of 0.21 l min⁻¹. The ALR was operated continuously twice for 21 days. The inlet substrate concentration was 2.04 x 10⁻¹ mol m⁻³. The flow rates employed were 1.5 x 10⁻⁹ m³ s⁻¹ in the first experimental run, and 2.5 x 10⁻⁹ m³ s⁻¹ in the second experiment. In both experiments reactor gel hold-up was 20 % (v/v). Biocatalyst gel load in the first experimental run was 53 kg m³ gel while in the second run 28 kg m³ gel was used. Beads were kept in the reactor by a Teflon sieve at the overflow of the ALR. Two samples of 1 ml were taken daily to monitor substrate and product concentration.

Model input parameter values

Initial and operational parameter values used for the model calculations were as follows: maximum substrate consumption rate, $V_{max} = 9.25 \times 10^{-8}$ mol kg s⁻¹; Michaelis–Menten constant, $K_m = 9.21 \times 10^{-2}$ mol m⁻³; effective substrate diffusion coefficient, $D_{eff} = 9.7 \times 10^{-12}$ m² s⁻¹; mean bead diameter, $d = 2.05 \times 10^{-3}$ m; inlet substrate concentration, $C_{si} = 2.04 \times 10^{-1}$ mol m⁻³; substrate feed rate = 1.5 x 10⁻⁹ m³ s⁻¹ and 2.5 x 10⁻⁹ m³ s⁻¹; reactor gel hold-up = 20 % (v/v); biocatalyst gel load = 28 kg m³ gel and 53 kg m³ gel; mass transfer coefficient in a liquid film around the beads, $k_{l,s} = 1.54 \times 10^{-6}$ m s⁻¹, zero-order decay rate, $k_d = 6.39 \times 10^{-4}$ h⁻¹. The steady-state measurements were taken after the ALR was operated continuously for 14 and 21 days.

Results and Discussion

Kinetic parameters

Kinetic experiments were carried out in shake flasks containing washed cells of *A. teichomyceticus* at a concentration of 20 g l^{-1} dry weight. Ten different initial concentrations ($0.02 - 1 \text{ mM}$) of antibiotic A40926 were used in two independent experiments. This was done intentionally in order to assure reproducibility and consistency of the data. The initial reaction rates were determined by linear regression and expressed as moles deacyl-A40926 formed per kg dry cell weight per second. The experimental data of A40926 concentrations vs. initial reaction rates from both experiments were fitted to the Michaelis-Menten equation. Figure 3 shows that there is a good agreement between the experimental data and the fitted curve. In Michaelis-Menten kinetics, the estimation of V_{max} and K_m is rather poor if the experiments are carried out only at low concentrations ($[S] \ll K_m$). It is therefore important to explore a wide range of concentrations. Our experimental data show that the substrate concentrations chosen for the kinetic experiments were appropriate, covering also the range where $[S] \gg K_m$ (Fig.3).

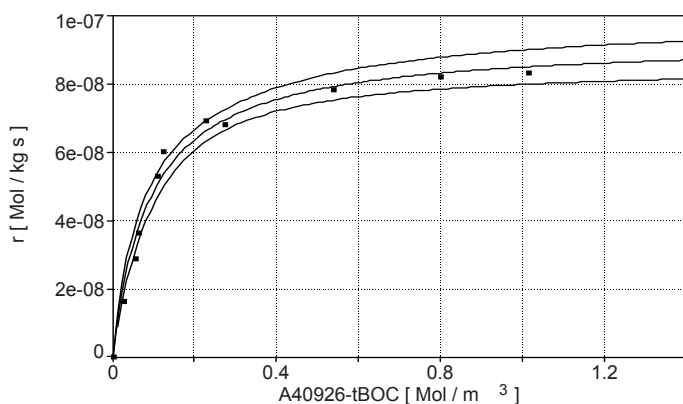


Figure 3 - The initial reaction rates as a function of A40926 concentration for the *Actinoplanes teichomyceticus* catalyzed deacylation. The experimental data are fitted to a Michaelis-Menten kinetic model (solid line corresponds to the best fit, dashed lines represent 95% confidence region)

The fitted model was found to explain 98 % (r_{adj}^2) of the total variation. The estimated parameters are shown in Table I. These values were used as intrinsic parameters for *A. teichomyceticus* cells immobilized in Ca-alginate.

Table I – Estimated kinetic constants for deacylation of A40926 by whole cells of *Actinoplanes teichomyceticus*

Parameter	Unit	Value	Min *	Max *
V_{max}	(mol kg s ⁻¹)	9.25×10^{-8}	8.54×10^{-8}	9.95×10^{-8}
K_m	(mol m ⁻³)	9.21×10^{-2}	6.79×10^{-2}	11.63×10^{-2}

* Min and max are 95% confidence limits for the parameter values

A40926 deacylation in ALR

Two experimental runs with ALR were carried out as described in Materials and Methods section. Conditions for two experiments were chosen in such a way that differences in estimated substrate bulk concentrations were large enough. In the first run higher biocatalyst gel load (53 kg m⁻³ gel) and lower feed rate (1.5×10^{-9} m³ s⁻¹) were chosen. In the second experiment the opposite was the case, *i.e.* lower biocatalyst gel load (28 kg m⁻³ gel) and higher feed rate (1.5×10^{-9} m³ s⁻¹) were chosen. Figure 4 shows steady-state antibiotic concentration after 14 and 21 days, together with model calculations. The difference in steady-state concentrations for two experimental runs was shown to be statistically significant as there were no overlap of the 95% confidence regions. That allowed us to examine performance of the ALR as well as model predictions under operational conditions of low and high relative conversion. Clearly, higher biocatalyst gel load and lower feed rate (*i.e.* higher residence time) in experimental run I (Fig.4) resulted in lower substrate bulk concentration and consequently higher relative conversion.

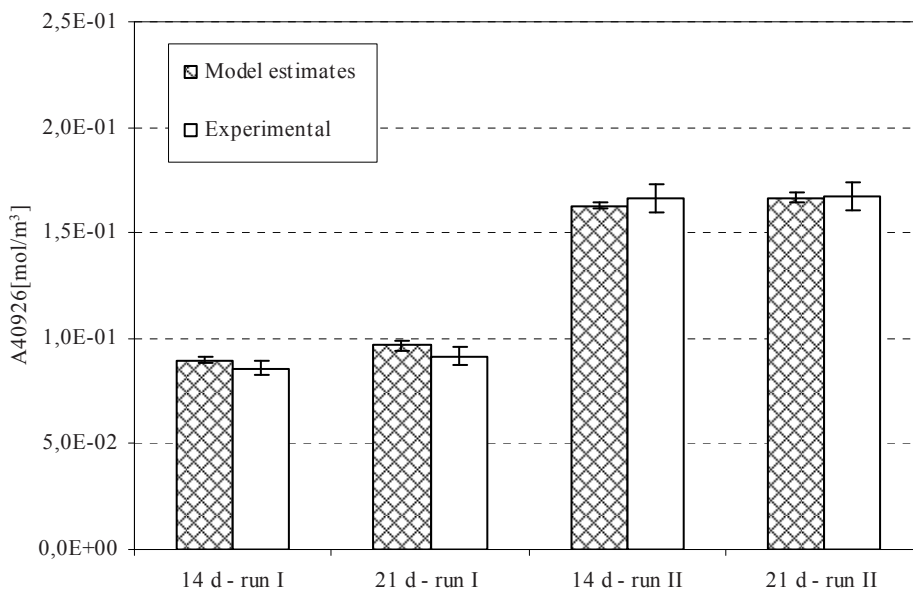


Figure 4 - Substrate bulk concentrations in both experimental runs after 14 and 21 days of operation. Empty bars represent observed values (error bars give 95% confidence region). Model estimates are given by cross-hatched bars (error bars give the range of estimates with $(k_{l,s} = 3.85 \times 10^{-7} \text{ m s}^{-1})$ and without film theory).

Substrate and product concentration profiles observed during the experiments suggested progressive linear loss of biocatalyst activity in the reactor. To account for the loss of activity in time the inactivation rate was calculated as a zero-order process. The decay rate k_d was estimated to be $6.39 \times 10^{-4} \text{ h}^{-1}$, which corresponds to the 19.3 % activity loss after two weeks at 28° C. For the model calculations shown in Figure 4, a correction of the maximal substrate conversion rate V_m was made using estimated inactivation rate. A number of days between start-up and steady-state measurement were taken into account.

Model estimates were obtained using method of De Gooijer *et al.*⁶. This calculation procedure considers internal diffusion and reaction in the beads with or without external mass-transfer resistance. In the immobilized biocatalyst systems, like the one described here, external mass-transfer can play a significant role in the overall mass-transfer resistance. The importance of the external mass-transfer can be evaluated by the Biot

number (Bi), which is defined as the ratio of the mass transfer resistance in the stagnant liquid film around the bead to the internal resistance found in the bead⁸. We used the procedure described by van't Riet and Tramper⁹ to estimate external mass-transfer resistance and considering the effective diffusion coefficient⁷, Biot number was calculated for given experimental conditions. The resulting value ($Bi = 163$) indicated that external mass-transfer resistance is negligible with respect to the internal mass-transfer resistance. Consequently, calculations were done excluding external mass-transfer resistance, and in order to give a range of model estimates using one quarter of the estimated value of $k_{l,s} = 1.54 \times 10^{-6} \text{ m s}^{-1}$. Figure 4 clearly shows that calculated estimates agreed well with the observed values in both experimental runs. This suggests that the model could be employed as adequate predictor for the deacylation of A40926 by immobilized biocatalyst.

Model simulations

A proposed model was used to simulate continuous deacylation of A40926 in order to examine the range of experimental conditions. The influent substrate concentration chosen is given in Materials and Methods and was the one used in our previous work⁵. Bead diameter and the gel load values were the same as in two experiments while the flow rate (i.e. residence time) was chosen as a variable that can be easily modified. Other parameter values were as given in Materials and Methods. In Figure 5, model estimates are given for the relative conversion as a function of the residence time using two experimental biocatalyst gel loads, namely 28 kg m^{-3} gel (dashed line) and 53 kg m^{-3} gel (solid line).

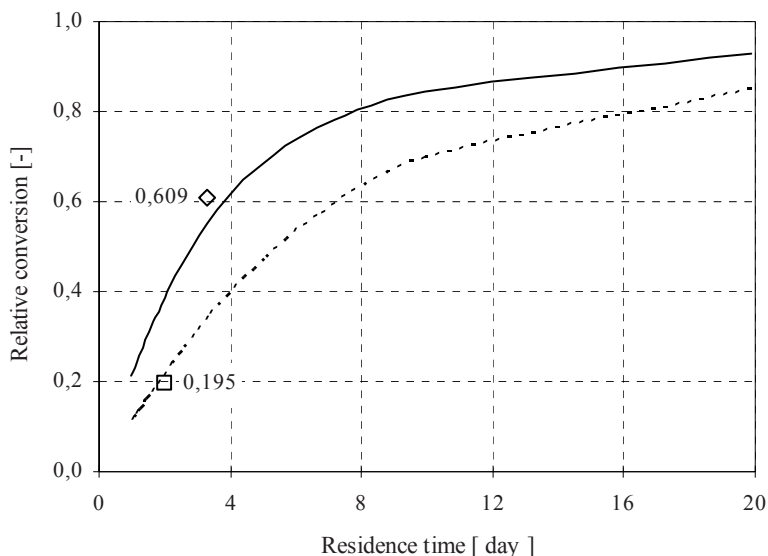


Figure 5 - Model estimates for the conversion of A40926 in the ALR as a function of residence time (solid line – biocatalyst gel load = $53 \text{ kg m}^{-3} \text{ gel}$, dashed line - biocatalyst gel load = $28 \text{ kg m}^{-3} \text{ gel}$). Experimental conversions are for run I (diamond symbol) and run II (square symbol).

It is clear that biocatalyst gel load has a significant effect on the relative conversion (dashed line remains below solid one over the whole range of residence times). This is more pronounced if shorter residence times are used. On the other hand, at increasing residence time, the conversion increases. Hence, a combination of parameters was examined in two experimental runs taking in consideration physical (i.e. biocatalyst gel load) and operational (i.e. residence time) limits of the system. Figure 5 shows the experimentally determined relative conversions of 19.5 and 60.9 % respectively. Clearly, increasing biocatalyst gel load and residence time concomitantly, a three fold higher conversion is obtained. The model estimates agree well with the experimental results of both experiments.

If a continuous deacylation process is to be developed, competitive advantage should be demonstrated over the established batch process. One of the most important aspects of the process, considering very high cost of the substrate (i.e. antibiotic), is the final conversion.

Thus, overall conversion in a continuous process should exceed 93%, usually obtained in a batch system [4]. In a single ALR this could be achieved (theoretically) only at very long and from the operational point of view impracticable residence times. This makes continuous processes based on a single reactor rather unattractive. Nevertheless, our results are encouraging. Considering kinetic characteristics, stability of the system and rather high conversion (> 60%) obtained with a single ALR, a cascade of bioreactors, could prove very attractive alternative to the existing batch process.

Conclusions

Continuous deacylation of A40926 by immobilized *Actinoplanes teichomyceticus* cells was evaluated in an air-lift loop-reactor. The deacylation kinetics were described by the Michaelis-Menten equation and kinetic parameters obtained. Using experimentally determined kinetic and diffusion⁷ parameters continuous deacylation was modelled applying procedures of De Gooijer *et al.*⁶. Inactivation of the biocatalyst as a function of time was observed during the experiments. Decay rate was found to be significant with respect to the duration of the experiments and was incorporated in the model calculations. System was operated continuously for three weeks with a constant conversion above 60%. Our current work is directed towards modelling and implementation of a series of ALR for the deacylation of A40926, and will be subject of our next paper.

References

1. Waltho JP, Williams DH, Selva E, Ferrari P. Structure elucidation of the glycopeptide antibiotic complex A40926. J Chem Soc Perkin Trans 1987;I: 2103-07.
2. Candiani G, Abbondi M, Borgonovi M, Romanò G, Parenti F. In-vitro and in-vivo antibacterial activity of BI 397, a new semi-synthetic glycopeptide antibiotic. J Antimicrobial Chemotherapy 1999;44:179-92.
3. Borghi A, Spreafico F, Beretta G, Ferrari P, Goldstein BP, Berti M, Denaro M, Selva E. Deacylation of the glycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* ATCC 31121. J Antibiotics 1996; 49: 607-09.
4. Carrelli A, Borghi A, Carrano L, Gastaldo L, Marinelli F. Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells. J Industrial Microbiology 1995; 15: 429-33.
5. Jovetić S, Tramper J, Marinelli F. Biotransformation of the lipoglycopeptide antibiotic A40926 with immobilized cells of *Actinoplanes teichomyceticus* - in situ supply of nutrients. Enzyme Microb Technol 1998; 22: 117-21.
6. De Gooijer CD, Hens HJH, Tramper J. Optimum design for a series of continuous tank reactors containing immobilized biocatalyst beads obeying intrinsic Michaelis-Menten kinetics. Bioproc Eng 1989; 4:153-58.
7. Jovetić S, Beftink HH, Tramper J, Marinelli F. Diffusion of (de)acylated antibiotic A40926 in alginate and carrageenan beads with or without cells and/or soybean meal. Enzyme Microb Technol 2001; 28: 510-14.
8. Wijffels RH, De Gooijer CD, Kortekaas S, Tramper, J. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan: Part 2. Model evaluation. Biotechnol Bioeng 1991; 38:232-240
9. Riet, K and Tramper, J. *Basic Bioreactor Design*. Marcel Dekker, New York, 1991, pp. 243-244

Chapter 5

Continuous biotransformation of glycopeptide antibiotic A40926 in a cascade of three airlift bioreactors using immobilized *Actinoplanes teichomyceticus* cells

Abstract

Immobilized cells of *Actinoplanes teichomyceticus* ATCC 31121 were used to selectively cleave the acyl group of A40926 yielding the deacylated form of the molecule. The feasibility of this particular biotransformation in a series of three perfectly mixed air-lift bioreactors with immobilized cells was examined. A continuously operated air-lift cascade was designed using a model for a series of reactors with immobilized biocatalyst beads obeying Michaelis-Menten kinetics. In independent experimental runs the cascade bioreactor system was operated continuously for 56 days with an overall conversion of 99%. Model estimates for reactor volumes and relative conversions were found to be in a good agreement with the experimental results.

Introduction

Glycopeptide antibiotics are a family of compounds of natural origin. They demonstrate a strong activity against Gram-positive aerobic and anaerobic bacteria and this led to the introduction into clinical practices of vancomycin and teicoplanin. A40926 is another member of the glycopeptide antibiotic family, which was discovered in our laboratories^{1,2}. It is produced by *Nonomuraea* sp. ATCC 39727 and it is the precursor for the semi-synthesis of dalbavancin, a novel second-generation glycopeptide agent³. Dalbavancin is currently in Phase III of clinical studies and it appears to be a potent antibiotic for the treatment of patients infected with the difficult-to-treat strains of MRSA (methicillin-resistant *Staphylococcus aureus*) and MRSE (methicillin-resistant *Staphylococcus epidermidis*)^{4,5}.

In recent years the rapid emergence of bacterial resistance to the existing glycopeptide antibiotics became an issue of great concern to public health and resulted in an ever-increasing clinical need for new and more potent anti-microbial agents⁶. In an R&D program to generate new and more active analogs of A40926 pursued in our laboratories, biotransformation combined with chemical modifications was attempted^{7,8,9}. The deacylated form of A40926 was proposed as an interesting molecule that can act as a starting point for the synthesis of novel structures. Deacylation of A40926 was successfully achieved with whole cells of *Actinoplanes teichomyceticus* ATCC 31121 and a batch process was developed^{10,11}. As a further development, we examined the feasibility of using immobilized *A.teichomyceticus* cells for this particular biotransformation¹². These results suggested that a continuous process with immobilized cells might be an interesting alternative to the established batch process. Consequently, the deacylation reaction was described with Michaelis-Menten equation, the kinetic parameters were determined, and a continuous processing with immobilized cells was attempted in a single airlift reactor (ALR)¹³. However, the high cost of the substrate (*i.e.* A40926) combined with a complex downstream process and final purification, demands for a process able to deliver a high conversion yield (> 95 %). In our continuous system based on a single reactor such a high conversion was never achieved and a series of bioreactors was suggested as an alternative.

Here we describe the design and the continuous deacylation in a cascade of three ALRs. To design a cascade the theoretical development and the calculation procedures of De Gooijer *et al.*¹⁴ were used. This model, based on the earlier work of Luyben and Tramper¹⁵, describes the simultaneous diffusion and conversion of substrate in the biocatalyst bead, giving an estimation of the substrate concentration profile in the bead. It is used in the design of perfectly mixed reactors in series, containing the immobilized biocatalyst obeying intrinsic Michaelis-Menten kinetics. In literature, the model was evaluated with immobilized enzymes and Bakker *et al.*¹⁶ described sucrose conversion with immobilized invertase in a series of bioreactors. Here, for the first time the model was evaluated in a series of bioreactors with immobilized whole aerobic cells. From practical point of view a short, three-vessel cascade is well suited when aerobic systems are studied, so we employed that set-up in our experiments. In the present contribution we used the model for the predictions and previously determined M-M kinetic parameters¹³ and effective diffusion coefficients¹⁷ were used in the calculations. Model estimates for reactor volumes and relative conversions in ALRs were compared with experimental results and the overall performance of the cascade is discussed.

Materials and Methods

Chemicals

Substrate, A40926 *tert*-BOC and the deacylated product, deacyl A40926 *tert*-BOC (in further text A40926 and deacyl A40926) were prepared in our laboratories as previously described¹².

Organism, Culture Conditions, and Immobilization Method

Actinoplanes teichomyceticus ATCC 31121 was maintained as a frozen vegetative stock at -80 °C and cultivated as described by Jovetić *et al.*¹³. Immobilization in 2% calcium alginate gel was carried out by a procedure previously described¹². Two batches of beads were prepared with gel loads of 28 and 53 kg of dry cell weight per m³ of gel, respectively. Leitz Wetzlar stereoscope (10X magnification) was used to measure the diameter of the beads. From a sample of 150 beads the average bead diameter ($d_{\text{mean}} \pm \text{SD}$) was determined. Values of 2.01 ± 0.09 mm and 2.05 ± 0.11 mm were found for the two batches of beads and used in model calculations.

Analyses

Substrate and product concentrations were determined by HPLC. Analytical method, sample preparation and calculation procedure used were as given by Jovetić *et al.*¹².

Cascade experiments

A series of three equal-sized airlift reactors (ALR) was assembled (a schematic representation of the cascade is given in Fig.1).

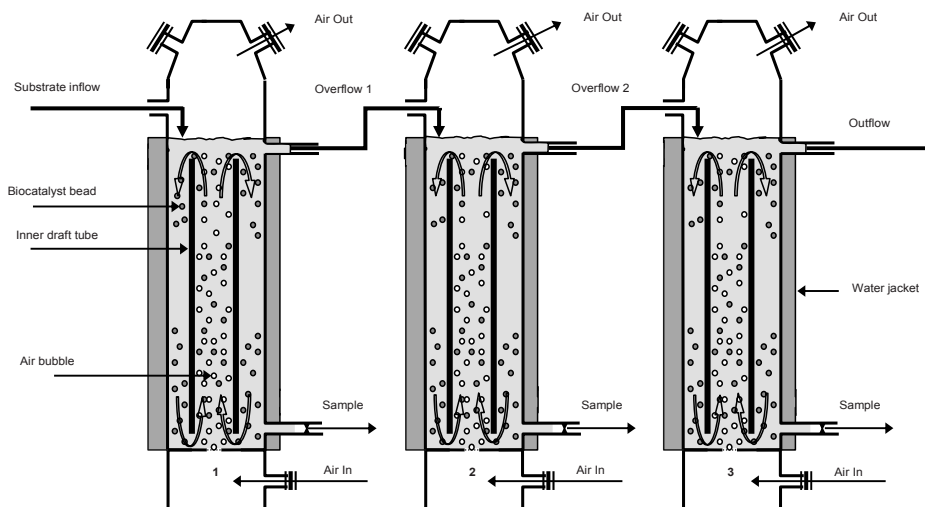


Figure 1 - Cascade of three air-lift reactors (cross-section view)

Internal diameter of each vessel was 0.04 m and liquid height 0.34 m. The working volume of each single reactor was 0.43 L, giving a total working volume of the cascade of 1.29 liters. Operational temperature was 28 °C and airflow rate 0.21 L min⁻¹. The substrate, antibiotic A40926, was dissolved in 20 mM Tris-HCl buffer (pH 7.0) and fed continuously. The inlet substrate concentration chosen was $2.04 \times 10^{-1} \text{ mol m}^{-3}$ as used also in our previous experiments of biotransformation^{12,13}. Reactor gel hold-up was 20 % (v/v), a value commonly used in heterogeneous biocatalysis. To choose appropriate residence times in the cascade, several preliminary experiments were executed using two batches of biocatalyst beads and different substrate flow rates. At this early stage model estimates for a wide range of flow rates (*i.e.* residence times) were used in order to facilitate choice of operational conditions. On the basis of model estimates and preliminary experimental runs two distinct operational regimes were chosen for evaluation, namely Run A and Run B. In both runs the cascade was operated continuously for 56 days. In Run A, the flow rate employed was $1.5 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ and the biocatalyst gel load was 53 kg m⁻³ gel. In Run B, the flow rate was $2.5 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ and the biocatalyst gel load was 28 kg m⁻³ gel. Duplicate samples of 1 ml bulk liquid were taken daily to monitor substrate and product concentration.

Model input parameters

Parameter values used for the model calculations were as follows: maximum substrate consumption rate, $V_{max} = 9.25 \times 10^{-8} \text{ mol kg s}^{-1}$ and Michaelis–Menten constant, $K_m = 9.21 \times 10^{-2} \text{ mol m}^{-3}$ ¹³; effective substrate diffusion coefficient, $D_{eff} = 9.7 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$ ¹⁷; mean bead diameter for the run A, $d_I = 2.01 \times 10^{-3} \text{ m}$; mean bead diameter for the run B, $d_{II} = 2.05 \times 10^{-3} \text{ m}$; Inlet substrate concentration, $C_{si} = 2.04 \times 10^{-1} \text{ mol m}^{-3}$; substrate feed rate $F_I = 1.5 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ (Run A) and $F_{II} = 2.5 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ (Run B); reactor gel hold-up $\varepsilon = 20 \%$ (v/v); biocatalyst gel load $X_I = 53 \text{ kg m}^{-3} \text{ gel}$ (Run A) and $X_{II} = 28 \text{ kg m}^{-3} \text{ gel}$ (Run B); first-order biocatalyst decay rate, $k_d = 5.4 \times 10^{-3} \text{ d}^{-1}$.

Results and Discussion

Choosing the cascade

In our previous work we studied a continuous deacylation in a single air-lift reactor¹³. Examining a range of conditions we managed to reach a maximum conversion of 61 %, quite distant from the at least 95 % needed. This was mainly due to the rather high M-M constant (K_m) of deacylation¹³ and to the fact that we used only one continuous reactor. Theoretically, a higher conversion can be achieved in a single reactor, but requires a very long and operationally impracticable residence time. To overcome this hurdle, a plug-flow mode of operation was contemplated. The main difficulty in operating a plug-flow reactor with immobilized aerobic cells is the need to supply oxygen. Thus, a cascade of airlift reactors was considered as an alternative. When several ($N \geq 3$) perfectly mixed vessels are used in series, plug-flow behavior can be approximated. ALRs used in our studies were small-scale vessels ($V < 1$ l) having a regular aspect ratio ($H/D < 9$), thus behaving as ideally mixed reactors (see next section). Hence, a cascade of three internal-loop airlift reactors was assembled and implemented as an approximation of an aerated plug-flow bioreactor.

Mixing in the cascade

When a cascade of bioreactors is operated in a continuous fashion (as described here) the liquid residence time in each reactor should largely exceed the mixing time, in order to prevent short cut flow of substrate between the single reactors. To evaluate mixing in airlifts, the concept of characteristic times was introduced and Verlaan showed¹⁸ that for airlift loop reactors:

$$T_{mix} = (4 \text{ to } 7) \times T_{circ} \quad (1)$$

where T_{mix} is the characteristic mixing time of the liquid phase (s) and T_{circ} is the characteristic liquid circulation time (s). Considering terminal rising velocity of the gas

bubbles in a liquid (approximately 0.25 m/s ¹⁹) and the small size of the reactors used in this study (liquid height of 0.34 m), characteristic circulation time was expected in the order of seconds. Thus, according to Eq. 1 the characteristic mixing time is estimated to be less than a minute. In the two experimental runs described here, the liquid residence times used were more than two orders of magnitude higher than the estimated mixing time, thus guaranteeing perfect mixing and no short-cut flows between reactors.

Continuous A40926 deacylation

A40926 is a very expensive substrate as it is used in biotransformation as highly purified antibiotic. Also downstream processing of the deacylated product is costly and greatly dependent on the degree of conversion. Because of these considerations, a main goal of the continuous deacylation process was to maximize substrate conversion, aiming at 99% or more. To achieve the desired conversion in a continuous biotransformation, substrate feed rate is often the parameter of choice that is easy to modify. In several pilot experiments with the cascade, different substrate flow rates were evaluated using two batches of biocatalyst beads. For each flow rate the relative conversions in the outflow of the cascade was determined after steady state was achieved. In Table I experimental conversions observed in these experiments are given. Clearly, residence times longer than 2 days are required for the 99% conversion to be achieved with a biocatalyst gel load of 53 kg of dry cell weight per m^3 of gel.

Table I – Experimental conversions of A40926 (expressed in %) in the outlet flow of the cascade as a function of residence time and two biocatalyst gel loads

Residence time [days]	Biocatalyst gel load [kg m^{-3} gel]	
	28	53
1.3	39	62
1.7	55	78
2.1	61	85

Besides a high conversion, a second objective of our work was model evaluation, and in

order to evaluate model predictions adequately two distinct operational regimes were required that will provide large enough differences in relative conversions. Moreover, employment of different biocatalyst gel loads was preferred as additional model parameter variations. On the basis of these considerations and pilot experimental results, model calculations were used to set biocatalyst gel load and substrate feed rate parameter values for further experiments. Two different experimental conditions were chosen. High biocatalyst gel load / low substrate feed rate regime (Run A: 99 % theoretical conversion in model calculations) and low biocatalyst gel load / high substrate feed rate (Run B: 67 % theoretical conversion in model calculations). Under these conditions two 56-day runs, namely run A and run B, were executed as described in the Materials and Methods section. Figure 2 shows the experimental values of the relative conversions (expressed as the ratio of product concentration and inlet substrate concentration) in all three reactors during experimental run A. It can be seen that experimentally observed relative conversions in the outflow of the cascade were between 95 and 99 % throughout the experiment. This compares favourably to the established batch process where overall conversion achieved is usually $\leq 90\%$ [11]. Two weeks after the start of the experiment a progressive decrease in the relative conversions was observed suggesting a constant loss of catalytic activity. Usually such trend is described by first-order kinetics. Accordingly, the decay rate, k_d , was estimated to be $5.4 \times 10^{-3} \text{ d}^{-1}$, and taken into account in the model calculations. After six weeks of continuous operation (day 42), the relative conversion in the outflow fell below 95 % (Fig. 2). In order to check whether, with the residual biocatalyst activity present in the system, the conversion could be brought back to the desired level (i.e. 99%), the substrate feed rate was adjusted, according to the model estimates, to $1 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ (see next section)

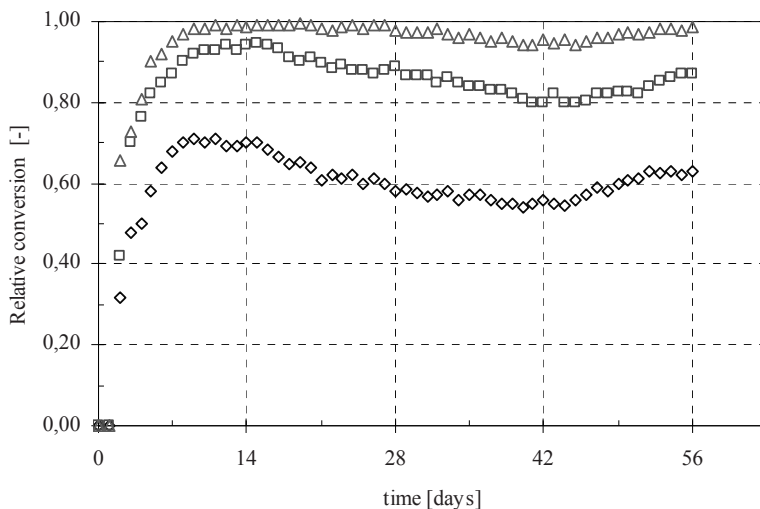


Figure 2 - Experimental data for relative conversions in the cascade during the first run (ALR 1 - diamond, ALR 2 – square, ALR 3 – triangle)

After a new steady state was reached (day 54), the residual substrate concentration in the outflow of the cascade fell to 1 % of the inflow value. In experimental run B a low catalyst load / high feed rate regime was applied. As predicted, only 64 % relative conversion was achieved under these conditions (data not shown). Continuous operation, conversions of 99% and even higher (as achieved in run A), and facilitated recovery are all elements of competitive advantage for a biotransformation process. These facts, combined with the appropriate process optimization, could make a continuous deacylation with immobilized cells an interesting and economically viable alternative to the established batch process.

Model estimates

A model developed earlier in our laboratory¹⁴ was used to design the cascade of ALRs. The calculation procedure considers internal diffusion/reaction rate of the substrate in the immobilized biocatalyst beads suspended in ideally mixed reactors. By setting the desired degree of substrate conversion and starting with the last reactor in the cascade, the

dimensionless concentrations (*i.e.* conversion) were calculated for each reactor. Consequently, the holding times and, as a result, the volumes of reactors were calculated. Initial and operational values used in the model simulations are given in Materials and Methods section. The experimental conversions were determined daily and the biocatalyst decay rate (k_d) was taken into account in the model calculations. The experimentally observed values and model output data are given in Table II. It clearly shows that calculated estimates agree well with the observed values in both experimental runs. When the conversion in the outflow fell to 95 % (day 42, Fig. 2) model calculations were used to adjust the flow rate so that the expected conversion is 99%. After steady state was reached with a new flow rate experimentally observed conversions indeed coincided with the predicted values (see previous section). This suggests that, considering the range of the experimentally examined conditions, the proposed model can be employed as a tool to design and also adequately interpret the deacylation of A40926.

Table II – Experimental data and model calculated values for the first and the second run, obtained after 56 days of operation.

Parameter	Run A		Run B	
	Experimental	Model	Experimental	Model
$V_{\text{tot}} [10^{-3} \text{ m}^3]^a$	1.29	1.31	1.29	1.30
$C_{\text{rel}} 1 [\%]^b$	63	62	28	30
$C_{\text{rel}} 2 [\%]^b$	87	90	50	52
$C_{\text{rel}} 3 [\%]^b$	99	99	64	67

^a total working volume of three air-lift reactors

^b relative conversions (expressed as ratio of product concentration to inlet substrate concentration) in each reactor of the cascade

As mentioned before, the model considers heterogeneous biocatalysis where the diffusion and the conversion of the substrate in the immobilized biocatalyst bead simultaneously occur. Concomitant diffusion/reaction situations imply a progressive decrease of the substrate concentration in the beads. By implementing the concept of the Thiele modulus²⁰

in the calculation procedure, the model can estimate the effectiveness factors and the substrate concentration in the bead. In Figure 3, a concentration profile for a biocatalyst bead in the last airlift reactor of the experimental run A is shown.

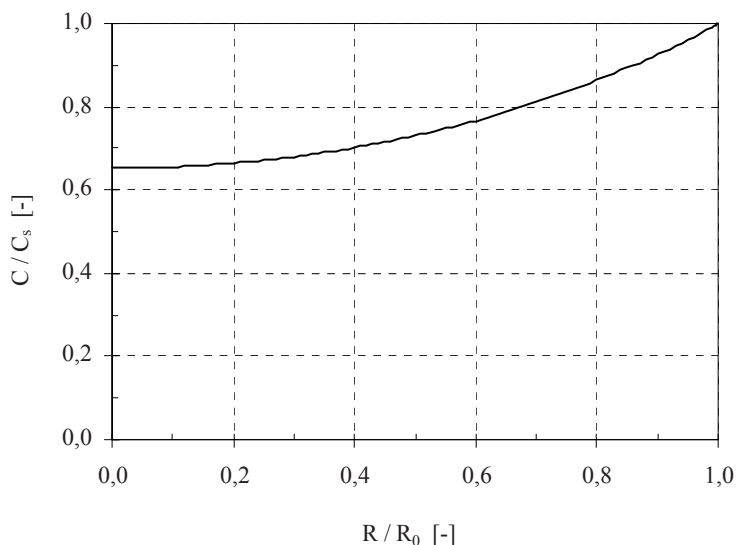


Figure 3 - Estimated substrate concentration profile in a biocatalyst bead present in the last air-lift reactor during the first experimental run (R/R_0 –normalized bead radius; C/C_s – substrate concentration normalized to the substrate concentration on the surface of the bead)

The calculated profile suggests that all of the biocatalyst present in the bead is in contact with the substrate. It is also evident that the substrate concentration in the centre of the bead is about 65 % of the substrate bulk concentration. In this case overall effectiveness factor was calculated to be 0.94 and considering that the K_m value is quite lower than the substrate concentration in the centre of the bead, it is clear that A40926 diffusion in the bead is not limiting the biotransformation rate to a high extent.

Conclusions

Using immobilized *Actinoplanes teichomyceticus* cells a continuous biotransformation of A40926 was investigated in a series of three airlift loop-reactors. To design the cascade and evaluate a range of experimental conditions, a model developed by De Gooijer *et al.*¹⁴ was used. Distinct operational regimes were examined in independent experimental trials. In both runs the cascade system was operated continuously for two months. The model estimates obtained for reactor volumes and substrate conversions agreed well with the experimental results. Under the operational regime of high biocatalyst gel load and low substrate feed rate, 95-99% conversions were achieved and maintained throughout the two months of operation. These results demonstrate the feasibility of the continuous biotransformation of A40926 at laboratory scale.

If the new process is to be brought to industrial scale, the competitiveness of the continuous deacylation must be demonstrated. A very high final conversion combined with an extensive continuous operation time represents a strong competitive advantage of the continuous process over the established batch deacylation. These parameters might even be improved by increasing the biocatalyst gel load and/or the reactor gel hold-up (conditions still physically possible). Thus, there is a ground for the further improvement of the continuous process by suitable combination of operational parameters. For that purpose, the proposed model was demonstrated to be a powerful design tool and could be used for adequate scale-up and subsequent process optimization.

References

1. Goldstein BP, Selva E, Gastaldo L, Berti M, Pallanza R, Ripamonti F, Ferrari P, Denaro M, Arioli V, Cassani G. A40926, a new glycopeptide antibiotic with anti-*Neisseria* activity. *Antimicrob Agents Chemother.* 1987;31:1961-1966.
2. Walto JP, Williams DH, Selva E, Ferrari P. Structure elucidation of the glycopeptide antibiotic complex A40926. *J Chem Soc Perkin Trans.* 1987;2103-2107.
3. Goldstein BP, Candiani G, Arain TM, Romano G, Ciciliato I, Berti M, Abbondi M, Scotti R, Mainini M, Ripamonti F, . Antimicrobial activity of MDL 63,246, a new semisynthetic glycopeptide antibiotic. *Antimicrob Agents Chemother.* 1995;39:1580-1588.
4. Seltzer E, Dorr MB, Goldstein BP, Perry M, Dowell JA, Henkel T. Once-weekly dalbavancin versus standard-of-care antimicrobial regimens for treatment of skin and soft-tissue infections. *Clin Infect Dis.* 2003;37:1298-1303.
5. Goldstein EJ, Citron DM, Merriam CV, Warren Y, Tyrrell K, Fernandez HT. In vitro activities of dalbavancin and nine comparator agents against anaerobic gram-positive species and corynebacteria. *Antimicrob Agents Chemother.* 2003;47:1968-1971.
6. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest.* 2003;111:1265-1273.
7. Malabarba A, Ciabatti R, Scotti R, Goldstein BP, Ferrari P, Kurz M, Andreini BP, Denaro M. New semisynthetic glycopeptides MDL 63,246 and MDL 63,042, and other amide derivatives of antibiotic A-40,926 active against highly glycopeptide-resistant VanA enterococci. *J Antibiot (Tokyo).* 1995;48:869-883.
8. Hermann R, Ripamonti F, Romano G, Restelli E, Ferrari P, Goldstein BP, Berti M, Ciabatti R. Synthesis and antibacterial activity of derivatives of the glycopeptide antibiotic A-40926 and its aglycone. *J Antibiot (Tokyo).* 1996;49:1236-1248.
9. Pavlov AY, Preobrazhenskaya MN, Malabarba A, Ciabatti R. Synthesis and antibacterial activity of derivatives of the glycopeptide antibiotic A-40926 N-alkylated at the aminoglucuronyl moiety. *J Antibiot (Tokyo).* 1998;51:525-527.
10. Borghi A, Spreafico F, Beretta G, Ferrari P, Goldstein BP, Berti M, Denaro M, Selva E. Deacylation of the glycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* ATCC 31121. *J Antibiot (Tokyo).* 1996;49:607-609.
11. Carrelli A, Borghi A, Carrano L, Gastaldo L, Marinelli F. Biotransformation of the lipoglycopeptide antibiotic A40926 by *Actinoplanes teichomyceticus* cells. *J Industrial Microbiology.* 1995;15:429-433.
12. Jovetić S, Tramper J, Marinelli F. Biotransformation of the lipoglycopeptide antibiotic A40926 with immobilized cells of *Actinoplanes teichomyceticus* - in situ supply of nutrients. *Enzyme Microb Technol.* 1998;22:117-121.

13. Jovetić S, de Bresser L, Tramper J, Marinelli F. Deacylation of antibiotic A40926 by immobilized *Actinoplanes teichomyceticus* cells in an internal-loop air-lift bioreactor. *Enzyme Microb Technol.* 2003;32:546-552.
14. De Gooijer CD, Hens HJH, Tramper J. Optimum design for a series of continuous tank reactors containing immobilized biocatalyst beads obeying intrinsic Michaelis-Menten kinetics. *Bioproc Eng.* 1989;4:153-158.
15. Luyben KCAM, Tramper J. Optimal design for continuous stirred tank reactors in series using Michaelis-Menten kinetics. *Biotechnol Bioeng.* 1982;24:1217-1220.
16. Bakker WA, Knitel JT, Tramper J, De Gooijer CD. Sucrose conversion by immobilized invertase in a multiple air-lift loop bioreactor. *Biotechnol Prog.* 1994;10:277-283.
17. Jovetić S, Beftink HH, Tramper J, Marinelli F. Diffusion of (de)acylated antibiotic A40926 in alginate and carrageenan beads with or without cells and/or soybean meal. *Enzyme Microb Technol.* 2001;28:510-514.
18. Verlaan P, Van Eijs AMM, Tramper J, van't Riet K, Luyben KChAM. Estimation of axial dispersion in individual sections of an airlift-loop reactor. *Chem Eng Sci.* 1989;44:1139-1146.
19. Heijnen JJ, Riet Kv. Mass transfer, mixing and heat transfer phenomena in low viscosity bubble column reactors. *Chem Eng J.* 1984;28:B21-B42.
20. van't Riet K, Tramper J. *Basic Bioreactor Design.* Marcel Dekker. New York. 1991

Chapter 6

Factors influencing cell fatty acid composition and A40926 antibiotic complex production in *Nonomuraea* sp. ATCC39727

Abstract

A40926 is a glycopeptide antibiotic complex consisting of several structurally related factors. It is produced by fermentation of *Nonomuraea* sp. ATCC 39727 and the complex components differ in the structure of the fatty acid moiety linked to the aminoglucuronic acid unit. In previous work we observed that the production of single factors in glycopeptide antibiotic complexes, could be selectively enhanced by the addition of suitable precursors to the culture medium. In this contribution we examine the effects of branched amino acid addition to fermentation of *Nonomuraea* sp. in a chemically defined minimal medium. The changes in the composition of cell fatty acids correlate to the fatty acid distribution within the A40926 complex in diverse cultivation conditions. *Nonomuraea* sp. prefers isobutyric, butyric and propionic acids as initiators of fatty acid biosynthesis. The relative amount of the produced fatty acids is significantly influenced by the availability of intermediates or final products from the amino acid catabolic pathways. Antibiotic complex composition closely reflects the cell fatty acid pattern, in agreement with the assumption that the antibiotic fatty acid moieties are synthesized by shortening the chain of cell fatty acids.

Introduction

Our interest in fatty acid metabolism in *Nonomuraea* ATTC 39727 derives from the observation of the close structural relationship between cell fatty acids and the shorter fatty acid moieties, which characterize the single components in the antibiotic complex produced by the strain. *Nonomuraea* cultures produce antibiotic A40926⁶, which is the starting material for the synthesis of dalbavancin, a clinically effective antibacterial antibiotic⁵. A40926 is a complex comprised of several structurally related factors (Fig 1). Complex components are known to differ in the structure of the fatty acid moiety (R1) linked to the glucuronic acid residue of the molecule^{19,20}. Main components of the complex are A₀ (R1 = *iso*-C11:0), A₁ (R1 = *n*-C11:0), B₀ (R1 = *iso*-C12:0) and B₁ (R1 = *n*-C12:0).

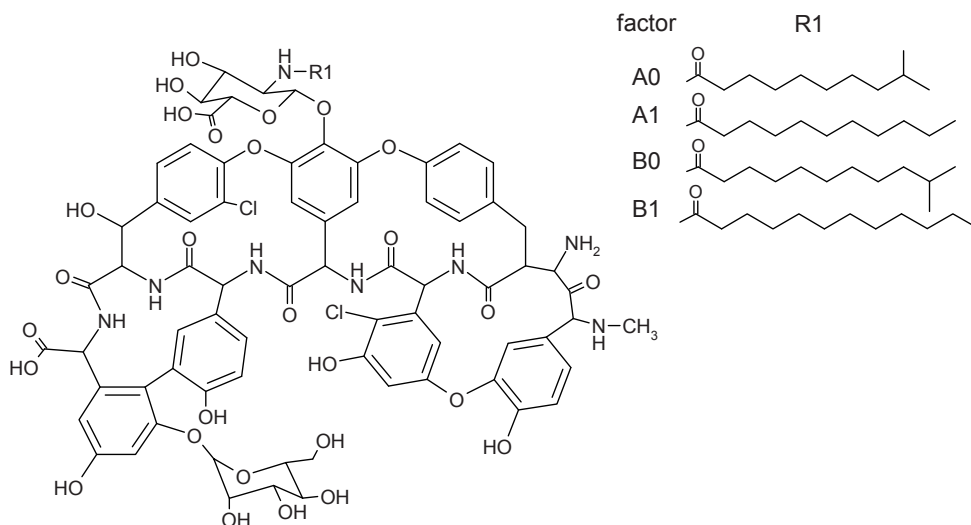


Figure 1 - A40926 chemical structure. Complex components differ in the acyl moieties substituted in the position R1 of the molecule.

The origin of the fatty acids constituting the acyl moieties of the glycopeptide molecules has been previously studied in the structurally closely related teicoplanin², A40926²⁰ and in the lipopeptide antibiotic ramoplanin³. Borghi *et al.*² studied factors affecting the normal and branched-chain acyl moieties of teicoplanin, and demonstrated that teicoplanin acyl

moieties are biosynthesized from longer cell fatty acids by shortening of the carbon chains. Since apparently the shortening is performed by the classical β -oxidation mechanism by loss of acetic acid units starting from the carboxyl end, the characteristic end terminal features (branched or linear) are conserved. Moreover the characteristic even or odd number of carbons is also conserved. The hypothesis was confirmed by the biosynthesis of novel teicoplanins bearing ten carbon chains with structural variations by adding to the culture medium C18 acids having corresponding structural features¹².

The biosynthesis of linear and branched fatty acids in prokaryotes has been elucidated in detail¹⁰. Branched chain fatty acids are synthesized using as chain initiator the coenzyme A esters of isobutyric acid (resulting in fatty acid with an even number of C atoms), isovaleric acid (resulting in fatty acid with an odd number of carbon atoms) or 2-methyl butyric acid (resulting in *ante-iso* fatty acid with an odd number of C atoms). These initiators can be easily provided by reactions, common to all branched amino acids catabolic pathways, consisting of the conversion by valine dehydrogenase of the amino acids into the corresponding α -ketoacids, namely α -ketoisovaleric, α -ketoisocaproic and 3-methyl- α -ketovaleric acids followed by oxidative decarboxylation. However, other ways of synthesizing the primers are known: we may note that all the above α -ketoacids are produced as intermediates in the pathway of branched amino acid synthesis, and that in *Streptomyces* spp. isobutyric acid can be formed by isomerisation of *n*-butyric acid⁸. The biosynthesis of branched chain and straight chain fatty acids in actinomycetes has been studied^{17,18} by adding predeuterated precursors to cultures grown on an amino acid rich medium. The nature and the origin of the chain initiators was demonstrated the same as that of bacilli. In the present contribution we examine the biosynthesis of fatty acids in *Nonomuraea* cultures grown in a chemically defined minimal medium containing ammonium ions as the only nitrogen source. In particular we assess the capacity of the strain of synthesizing the starters for the fatty acid chains, and the effect of biosynthetic precursors on both cell fatty acid composition and A40926 complex composition in the absence of potentially interfering nutrients usually present in rich media.

Materials and methods

Strains and Cultural Conditions

Nonomuraea sp. ATTC 39727 was maintained as a frozen vegetative stock at $-80\text{ }^{\circ}\text{C}$ in 15 % glycerol at a biomass concentration of approximately 0.08 g/ml DW (dry weight). Fermentation in the chemically defined minimal medium P150⁷ was carried out in shake-flasks. For vegetative seed culture preparations, a 500 ml baffled Erlenmeyer flask containing 100 ml seed medium described by Beltrametti *et al.*¹ was inoculated with one glycerol stock vial and incubated for 96 h at $28\text{ }^{\circ}\text{C}$ on a rotary shaker (200 rpm). Subsequently, seed culture was used for inoculating (at 10 % v/v) 100 ml aliquots of medium P150, and medium P150 supplemented with either 0.5 g/l of valine, 0.5 g/l of L-leucine or with 0.5 g/l of L-isoleucine, in 500 ml baffled Erlenmeyer flask. The shake-flasks were incubated at temperature of $28\text{ }^{\circ}\text{C}$ on a rotary shaker (200 rpm) and fermentation monitored for 96 h.

Analysis

Fermentation broth samples were collected at regular time intervals and analysed. Antibiotic production was monitored by HPLC analysis, as described by Beltrametti *et al.*¹ Biomass concentrations were determined by means of dry weight measurements. Glucose was analysed using Trinder assay (SIGMA Diagnostics, St. Louis, MO, USA). Inorganic phosphate was determined by malachite green method⁴. The indophenol direct method was used to assay ammonium concentrations⁹. After 72 h of fermentation, composition of the cell fatty acids was determined as fatty acid methyl esters derivatives (FAMES) by GC-MS method, previously described by Beltrametti *et al.*¹

Results and Discussion

Growth and production in minimal medium

The fermentation parameters of *Nonomuraea* sp. grown in the chemically defined P150 medium without amino acid supplements (i.e. reference conditions) are given in Fig. 2.

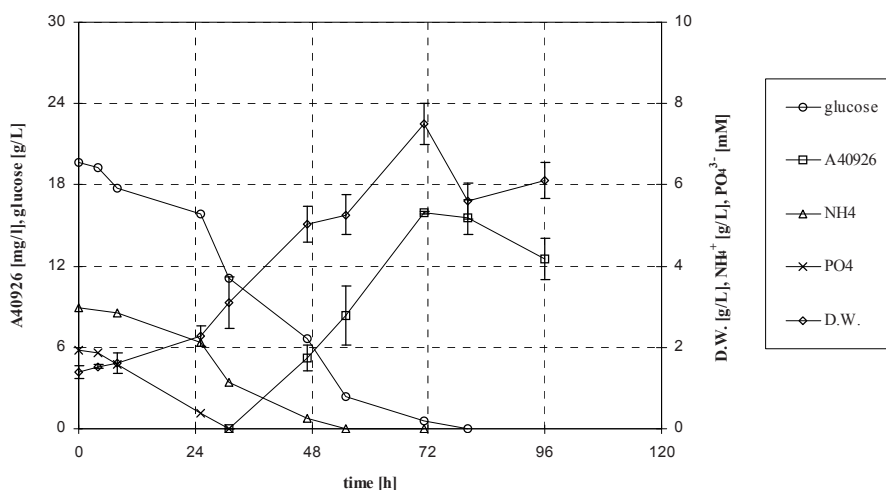


Figure 2 - Fermentation time course of *Nonomuraea* sp. grown in chemically defined P150 medium (error bars give a 95 % confidence region).

Data are mean values from three replicas of fermentations and each measurement was independently repeated three times. In contrast to what is commonly known for many secondary metabolites, the production of the A40926 antibiotic was found to be partly associated with the cell growth. This pattern was consistent with batch fermentation data previously reported by Gunnarson *et al.*⁷. Maximum antibiotic production of 16 mg/l was observed after 72 h of growth and it coincided with glucose exhaustion and concomitant end of biomass growth. At this culture time, samples of mycelium were collected and the composition of cell fatty acids was determined by GS-MS of their methyl esters (FAME). To study the effect of branched amino acids, the fermentation was repeated by adding to the medium 0.5 g/l of L-valine, L-leucine or L-isoleucine respectively and the composition of

the antibiotic complex as well as of the whole cell fatty acids was determined. Using relative abundance values, the percent contribution of the various FAMES to the fatty acid composition was calculated and given in Table1.

Table 1: Composition of cell fatty acids found under different fermentation and analysed as FAMES in GC-MS.

Rt [min]	Fatty acid	area%			
		P150	P150 + Val	P150 + Leu	P150 + Ile
13,94	<i>i 14:0</i>	-	0,64	0,61	0,56
14,96	<i>n 14:0</i>	4,44	2,76	2,78	1,82
16,65	<i>i 15:0</i>	1,16	3,52	5,02	2,69
17,61	<i>n 15:0</i>	1,68	6,54	1,35	10,33
18,63	<i>n 14:0 3-OH</i>	0,65	2,40	3,41	2,36
19,23	<i>i 16:0</i>	8,18	26,30	36,37	17,41
19,58	<i>16:1,9</i>	6,30	0,80	0,46	0,44
20,17	<i>n 16:0</i>	27,97	11,96	10,21	13,66
20,5	<i>n 15:0 3-OH</i>	-	-	17,38	1,23
21,25	<i>n 16:0 10 met</i>	13,16	1,87	3,33	1,28
21,72	<i>i 17:0</i>	0,78	0,76	1,78	-
21,96	<i>n 17:1,9</i>	1,21	3,36	0,99	4,59
22,62	<i>n 17:0</i>	1,20	4,87	1,05	6,41
23,59	<i>n 17:0 10 met</i>	8,40	23,98	8,02	32,21
24,28	<i>n 18:1,9</i>	2,92	0,91	0,40	-
24,73	<i>n 17:0 3-OH</i>	-	-	-	1,52
24,97	<i>n 18:0</i>	2,53	4,81	2,98	1,99
25,86	<i>n 18:0 10 met</i>	16,96	1,72	3,23	1,24
27,21	<i>n 19:0</i>	1,06	0,82	0,22	-
29,39	<i>n 20:0</i>	1,39	1,97	0,40	0,25

Cell fatty acid composition in minimal medium

In cells grown in medium P150 without supplements, linear acids with an even number of carbons predominated (Fig.3a and Table 1).

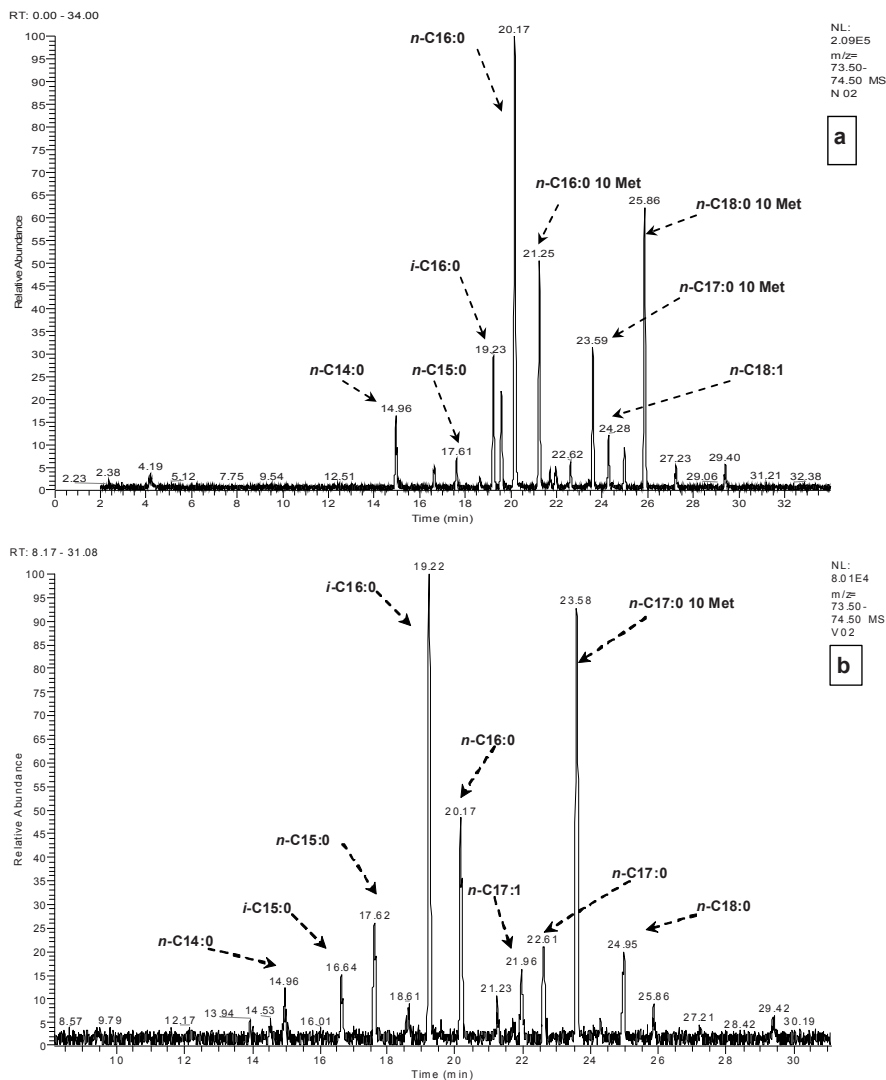


Figure 3 a&b - FAME GC-MS profiles for *Nonomuraea* sp. ATCC 39727 cell fatty acids from cultures grown in the chemically defined minimal medium P150 (a), and in medium P150 supplemented with 0.5 g/l of L-valine (b). Peaks relevant for the discussion are indicated.

The major component was *n*-C16:0. Summing up the contribution of the two linear hexadecanoic acids (*n*-C16:0 and *n*-C16:1), it overcame the 35% of the total. This suggests a large availability of butyrate, previously identified as the primer of such hexadecanoic acids either in bacilli or in actinomycetes^{17,18}. Among the linear acids primed by propionate, 10-methylheptadecanoic acid predominated (8,40%). This is not surprising since according to Kroppenstedt *et al.*¹¹ and Zhang *et al.*²¹, the presence of this acid is a typical taxonomical feature of *Nonomuraea* genus. Acids, whose synthesis is initiated by isobutyrate (*iso*-C16:0), contributed with the 8.18% to the total, and those initiated by isovalerate contributed with merely the 2.46%. Given the apparent large availability of butyrate, we can surmise that in our cells isobutyrate derived mainly from the butyrate isomerisation. Indeed, a pathway of butyrate synthesis from acetate and its conversion to isobutyrate was previously demonstrated in *Streptomyces* spp. by several studies (Han *et al.*⁸ and the literature therein quoted). In agreement with the fatty acid composition typical for *Nonomuraea* genus, *anteiso*- acids were not detected (Fig.3a and Table 1). This fatty acid composition of *Nonomuraea* cells grown in minimal medium, substantially differs from that reported by Zerilli *et al.*²⁰ for *Nonomuraea* cells grown in a complex medium. In that condition, the two predominant components were *iso*-hexadecanoic acid and 10-methyleptadecanoic acid. The first is primed by isobutyric acid, obviously provided by the partial catabolism of L-valine present in the culture medium. The second is primed by propionate, which is the end product of the catabolism of L-leucine and L-valine in many organisms¹³ including streptomycetes^{14, 16}.

L-Valine effect on cell fatty acid composition

In a previous paper from our laboratory, Beltrametti *et al.*¹ compared the pattern of fatty acid of cells grown in P150 medium with that of cells grown in P150 medium plus 0.75 g/l of L-valine. Beside a positive effect on antibiotic titre (see below), L-valine addition resulted in a substantial increase of *iso*-C16:0 acid coupled with a significant decrease in the relative amount of linear *n*-C16:0 acid. The results hereby presented (Fig.3b and Table 1) obtained with 0.5 g/l of L-valine, revealed that *iso*-C16:0 was in fact predominant, representing the 26.30 % of the total, whereas *n*-C16:0 contribution was decreased from

about 30% to about 12%. Another major effect of L-valine appeared to be the increase of *n*-C17:0-10-methyl acid from 8,40% to 23,98%. The biosynthesis of this chain is initiated by propionate, which is the end product of L-valine catabolism pathway. Other relevant amounts were those of *n*-C17:0 and *n*-C15:0 acids (4,87% and 6,54% respectively) also primed by propionate.

L-Leucine effect on cell fatty acid composition

When P150 medium was supplemented with 0.5 g/l of L-leucine or L-isoleucine, no significant changes in the fermentation trend or in the total antibiotic production were observed (data not shown). However upon addition of 0.5 g/l of L-leucine, important changes in cell fatty acid composition could be noticed (Fig.3c and Table 1). The contribution of *iso*-fatty acids with an odd number of carbon (*iso*-C15:0 and *iso*-C17:0) increased to 6.80% from the 1,84% observed in the un-supplemented medium. This was indeed a relatively small contribution, indicating that isovalerate is a poor substrate for the fatty acid synthase and therefore is largely available to be converted into isobutyric acid, the end product of L-leucine catabolic pathway. This is consistent with the unpredicted large increase of *iso*-C16:0, which represented the 36,37% of the total. It may be added that a secondary way of formation of isobutyric acid is possible. In fact, in many bacteria, L-leucine in relatively low concentrations inhibits the activity of α -isopropylmalate synthase, which catalyses the first step of leucine synthetic pathway, i.e. the conversion of α -ketoisovalerate into α -isopropylmalate. As a consequence, α -ketoisovalerate may accumulate and give rise, through oxidative decarboxylation, to isobutyric acid. Considering the other relevant acid it can be observed that the sum of the amounts of linear acids primed by propionate (*n*-C15:0, *n*-C17:0, *n*-C17:1, *n*-C17:0 10 Met), is approximately one third of the amount of *iso*-C16:0.

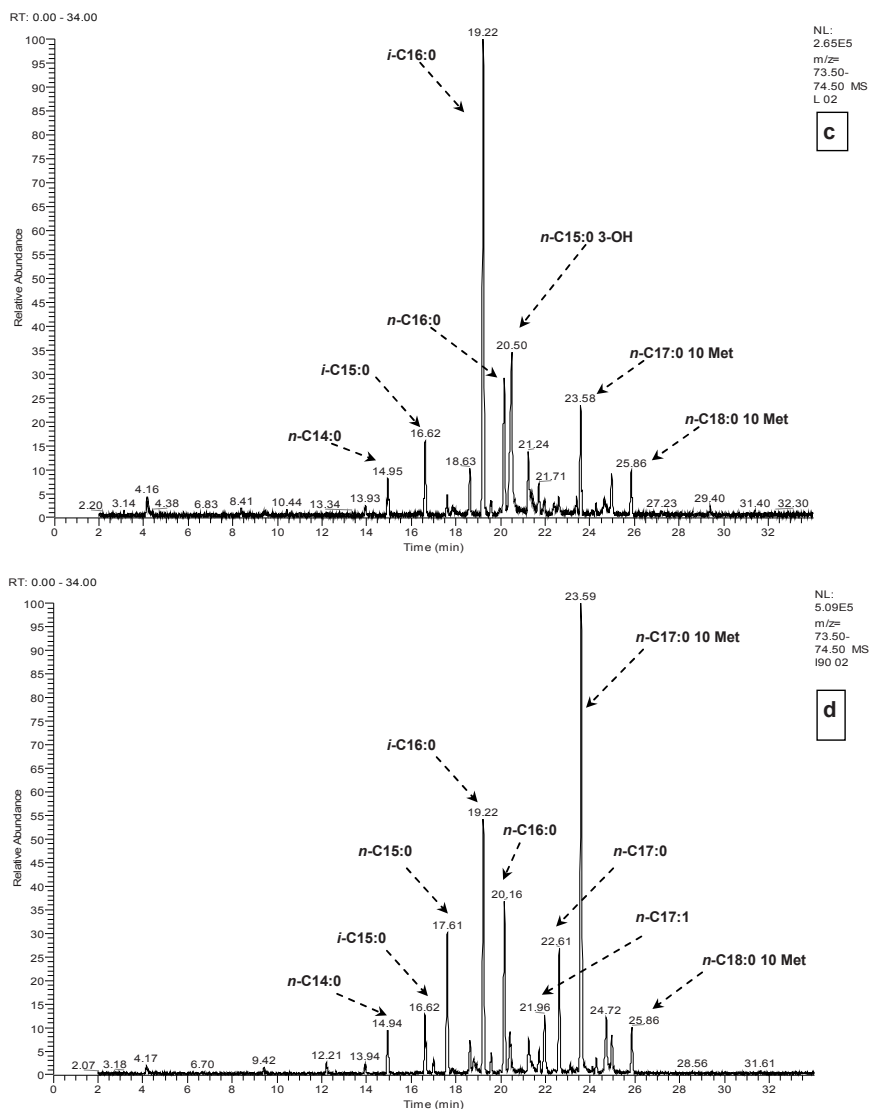


Figure 3 c&d - FAME GC-MS profiles for *Nonomuraea* sp. ATCC 39727 cell fatty acids from cultures grown in the chemically defined medium P150 supplemented with 0.5 g/l of L-leucine (c) or L-isoleucine (d). Peaks relevant for the discussion are indicated.

L-isoleucine effect on cell fatty acid composition

Addition of L-isoleucine to the fermentation medium resulted in a striking increase of odd-carbon linear fatty acids, namely *n*-C15:0 (11,14%), *n*-17:0 (6,91%), *n*-17:1 (4,95%) and *n*-

C17:0-10 met (34,35%) (Fig.3d and Table 1). This was understandable considering that propionyl-CoA and acetyl-CoA are the end products from L-leucine catabolic pathway. The first could be directly used for odd-carbon acid initiation. Acetyl-CoA, according to the above-mentioned pathway, could produce butyrate or isobutyrate and contribute to the synthesis of the observed *iso*-C16:0 and *n*-C16:0. No *anteiso*- fatty acids were observed, in spite of the demonstrated role of L-isoleucine as enhancer of their synthesis in many organisms. This suggests that the 2-methylbutyrate produced by L-isoleucine degradation is not recognized as a suitable substrate for fatty acid initiation in *Nonomuraea* sp.

A40926 complex composition

As mentioned before, the acyl moieties in the A40926 complex are believed to originate from the degradation of cell fatty acids. Analysis of the A40926 complex produced in the described conditions, confirmed this assumption. The relative abundance of A40926 main factors is given in Fig.4. In the fermentations carried out in medium P150 without supplements, the major antibiotic component was factor B₁, characterized by *n*-C12:0 acyl moiety. Its amount is 2.8 folds that of component B₀ (*iso*-C12:0) and is 12.2 and 20 folds that of components A₁ (*n*-C11:0) and A₀ (*iso*-C11:0), respectively. There is a qualitatively good correspondence between A40926 complex and the cell fatty acid pattern, considering that the linear hexadecanoic acid predominated among fatty acids, followed in the order by the *iso*-hexadecanoic, the heptadecanoic acids and then by the acids primed by isovalerate. Following the addition of L-valine (Fig.4), the change in fatty acid composition was reflected by the change in the antibiotic complex composition. Component B₀, characterized by an *iso*-C12:0 chain, became the major component of the antibiotic complex, in agreement with *iso*-C16:0 being the major component among cell fatty acids. The remarkable increase in *n*-C15:0 and *n*-C17:0 fatty acids was reflected by a major increase of A40926 factor A₁ (*n*-C11:0) synthesis. However, it appears evident that the 10-methyl-heptadecanoic acid, about as abundant as the *iso*-C16:0 acid, did not contribute to the formation of the *n*-C11:0 moiety of factor A₁. In fact, by the loss of three acetate units, the acid would be converted into the 4-methyl-undecanoic acid, not accepted as a substrate by the transacylase.

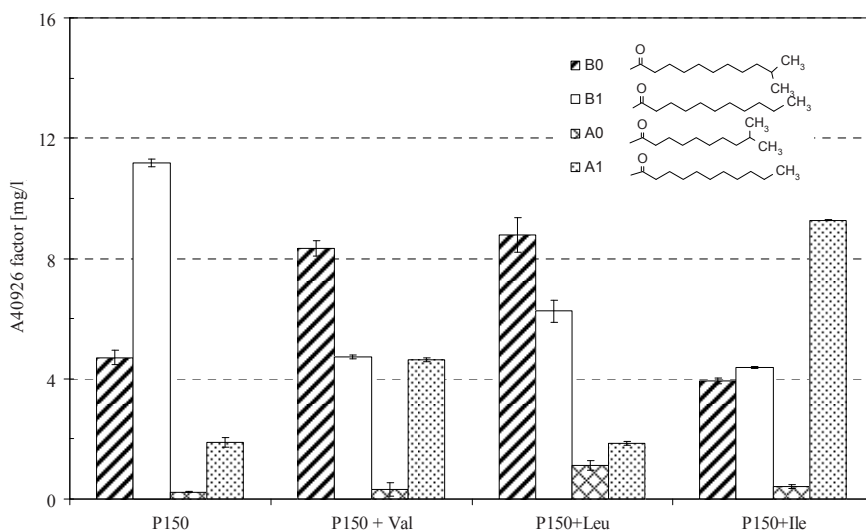


Figure 4 - Composition of the A40926 complex in the chemically defined minimal medium P150 and in medium P150 supplemented with 0.5 g/l of L-valine or L-leucine or L-isoleucine (error bars give a 95 % confidence region). The structures of the chains characterizing the factors are indicated.

The small amount of factor A0 is consistent with the minor contribution of *iso*-C15:0 to the cell fatty acids. In the case of L-leucine addition (Fig.4), as could be predicted from the fatty acid composition, factor B0 was the major constituent of the antibiotic complex. In spite of a five times increase with respect to reference condition, the contribution of factor A₀ (characterized by an *iso*-C11:0 chain) was still inferior to that of factor B1 (with a *n*-C12:0 chain), consistently with the ratios of the fatty acids from which they respectively derive. When L-isoleucine was added (Fig.4), 10-methyl-hexadecanoic was the predominant cell fatty acid. As discussed above, this acid could not contribute to the formation of the antibiotic fatty acid moieties. All together the other acids initiated by propionate represented the 23% of the total, versus the 17.91% of those initiated by butyrate and the 17.41% of those initiated by isobutyrate. This distribution was reflected in the composition of the antibiotic complex in which factor A1 predominated, followed in the order by factors B1 and B0.

In conclusion, when a single branched amino acid was added to the fermentation medium, the biosynthesis of fatty acids was influenced not only by the products of the initial steps of its catabolism, but also by the end products of the pathway, which, as it is the case of propionate, acted as the starters of specific fatty acids. The overall result depended on a combination of the biosynthetic starters' availability with the affinity of the starters for the synthase. It is evident that in *Nonomuraea* cells, three units, namely butyryl-CoA, isobutyryl-CoA and propionyl-CoA were about equally efficient in promoting the biosynthesis. Isovaleryl-CoA was accepted as a substrate to a lesser extent and 2-methylbutyryl-CoA was not recognized as a promoter. As a consequence in the case of L-isoleucine addition, no *anteiso* fatty acids were synthesized and the complete catabolism of the amino acid provided large amounts of propionyl-CoA, the primer of fatty acids with an even number of carbons. The case of L-leucine is also interesting since, due to the low affinity of isovalerate for the synthase, this intermediate was largely available for further degradation and was metabolized into isobutyryl-CoA, the end product of L-leucine catabolism. Therefore the prominent cell fatty acid was the *iso*-hexadecanoate, rather than the expected *iso*-C15 or *iso*-C17 acids.

In all the conditions examined, either in the absence or in presence of different amino acids, the relative proportion of the components in the antibiotic complex was qualitatively corresponding to the distribution of cell fatty acids. This could hardly be the case if the antibiotic acidic moieties were synthesized *ex novo* by a different synthase, and therefore constitutes a further confirmation of the origin of these moieties as degradation products from longer chain fatty acids. Sequencing of A40926 biosynthetic gene cluster¹⁵ revealed that genes devoted to fatty acid synthesis were not present in the indeed complete and highly organized cluster, further indicating that the acyl moieties introduced in the A40926 originate from cell fatty acid turnover. This observation may be of practical value since it indicates the possibility of altering the composition of the A40926 complex by the addition of suitable precursors and of obtaining new derivatives by supplementing the fermentation medium with sixteen- or eighteen-carbons acids bearing desired structural features.

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References

1. Beltrametti F, Jovetić S, Feroggio M, Gastaldo L, Selva E, Marinelli F (2004) Valine influences production and complex composition of glycopeptide antibiotic A40926 in fermentations of *Nonomuraea* sp. ATCC 39727. *J Antibiot* (Tokyo) 57: 37-44
2. Borghi A, Edwards D, Zerilli LF, Lancini GC (1991) Factors affecting the normal and branched-chain acyl moieties of teicoplanin components produced by *Actinoplanes teichomyceticus*. *J Gen Microbiol* 137: 587-92
3. Brunati M, Bava A, Marinelli F, Lancini G (2005) Influence of leucine and valine on ramoplanin production by *Actinoplanes* sp. ATCC 33076. *J Antibiot* (Tokyo) 58: 473-8
4. Bystrykh LV, Fernandez-Moreno MA, Herrema JK, Malpartida F, Hopwood DA, Dijkhuizen L (1996) Production of actinorhodin-related "blue pigments" by *Streptomyces coelicolor* A3(2). *J Bacteriol* 178: 2238-44
5. Candiani G, Abbondi M, Borgonovi M, Romano G, Parenti F (1999) In-vitro and in-vivo antibacterial activity of BI 397, a new semi-synthetic glycopeptide antibiotic. *J Antimicrob Chemother* 44: 179-192
6. Goldstein BP, Selva E, Gastaldo L, Berti M, Pallanza R, Ripamonti F, Ferrari P, Denaro M, Arioli V, Cassani G (1987) A40926, a new glycopeptide antibiotic with anti-*Neisseria* activity. *Antimicrob Agents Chemother* 31: 1961-1966
7. Gunnarsson N, Bruheim P, Nielsen J (2003) Production of the glycopeptide antibiotic A40926 by *Nonomuraea* sp. ATCC 39727: influence of medium composition in batch fermentation. *J Ind Microbiol Biotechnol* 30: 150-6
8. Han L, Reynolds KA (1997) A novel alternate anaplerotic pathway to the glyoxylate cycle in streptomycetes. *J Bacteriol* 179: 5157-64
9. Huizenga JR, Teelken AW, Tangerman A, de Jager AE, Gips CH, Jansen PL (1998) Determination of ammonia in cerebrospinal fluid using the indophenol direct method. *Mol Chem Neuropathol* 34: 169-77.
10. Kaneda T (1977) Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev* 41: 391-418
11. Kroppenstedt RM, Stackebrandt E, Goodfellow M (1990) Taxonomic revision of the actinomycete genera *Actinomadurea* and *Microtetraspora*. *System Appl Microbiol* 13: 148-60
12. Lazzarini A, Borghi A, Zerilli LF, Ferrari P, Colombo L, Lancini GC (1997) Novel teicoplanins by directed biosynthesis. *J Antibiot* (Tokyo) 50: 180-3
13. McMurry J, Begley T (2005) *The Organic Chemistry of Biological Pathways*. Roberts and Company Publisher, Englewood

14. Navarrete RM, Vara JA, Hutchinson CR (1990) Purification of an inducible L-valine dehydrogenase of *Streptomyces coelicolor* A3(2). *J Gen Microbiol* 136: 273-81.
15. Sosio M, Stinchi S, Beltrametti F, Lazzarini A, Donadio S (2003) The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *Nonomuraea* species. *Chem Biol* 10: 541-549
16. Tang L, Zhang YX, Hutchinson CR (1994) Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in *Streptomyces ambofaciens* and *Streptomyces fradiae*. *J Bacteriol* 176: 6107-19.
17. Wallace KK, Bao ZY, Dai H, Digate R, Schuler G, Speedie MK, Reynolds KA (1995) Purification of crotonyl-CoA reductase from *Streptomyces collinus* and cloning, sequencing and expression of the corresponding gene in *Escherichia coli*. *Eur J Biochem* 233: 954-62
18. Wallace KK, Zhao B, McArthur HA, Reynolds KA (1995) In vivo analysis of straight-chain and branched-chain fatty acid biosynthesis in three actinomycetes. *FEMS Microbiol Lett* 131: 227-34
19. Waltho JP, Williams DH, Selva E, Ferrari PJ (1987) Structure elucidation of the glycopeptide antibiotic complex A40926. *Chem Soc Perkin Trans 1*: 2103~2107
20. Zerilli LF, Edwards DM, Borghi A, Gallo GG, Selva E, Denaro M, Lancini GC (1992) Determination of the acyl moieties of the antibiotic complex A40926 and their relation with the membrane lipids of the producer strain. *Rapid Commun Mass Spectrom* 6: 109~114.
21. Zhang Z, Wang Y, Ruan J (1998) Reclassification of *Thermomonospora* and *Microtetraspora*. *Int J Syst Bacteriol* 48 Pt 2: 411-22

Chapter 7

General discussion

Introduction

This thesis focuses on the biotransformation of glycopeptide A40926 by immobilized cells of *Actynoplanes teichomyceticus* to produce a deacylated derivative. Deacyl-A40926 is used as a basic scaffold for medicinal chemistry modifications aiming to expand and improve the spectrum and/or potency of semisynthetic second-generation glycopeptide antibiotics. These antibiotics are today (still) considered as last line of defence against one of the most important human health threats, Multi-Resistant *Staphylococcus aureus*. A40926 is a natural antibiotic found in the fermentation broths of *Nonomuraea* sp. (a rare *Actynomcete* isolated from soil) and it demonstrates strong inhibitory activity against Gram-positive bacteria and *Neisseria gonorrhoea*. Like many other natural antibiotics, A40926 is a secondary metabolite of a quite complex structure. It is a mixture of several structurally very closely related compounds (called factors) that only differ in the type of the substituent present in the acyl-chain moiety of the molecule (Fig. 1). The origin and some aspects of these structural differences are addressed in Chapter 6, suggesting that novel derivatives might also be obtained by directed biosynthesis.

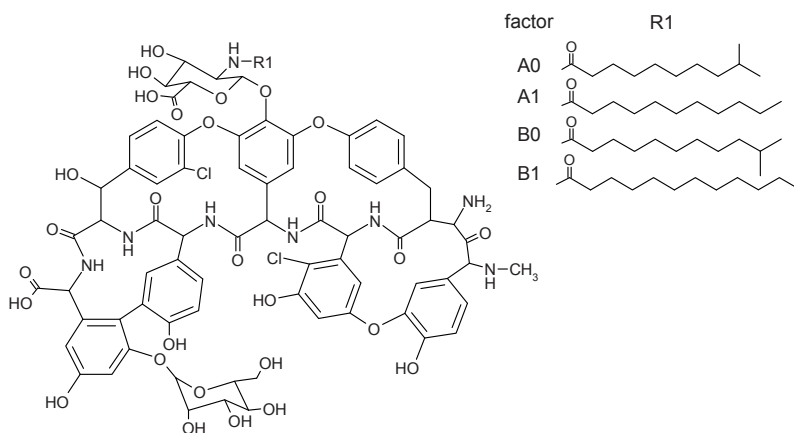


Figure 1 - A40926 chemical structure. Components of A40926 (factors A₀, A₁, B₀ and B₁) differ in the acyl chain substituent in the position R1 of the molecule.

The multifaceted structure makes A40926 a challenging scaffold for derivatization. This combined with an excellent activity against Gram-positive *Staphylococcus aureus* offer a unique opportunity for the discovery and the development of new semisynthetic glycopeptide antibiotics. However, chemical modification, such as deacylation, of a complex glycopeptide molecule is an almost impossible task and the solutions are often sought in the form of biotransformation. We employed *A. teichomyceticus* cells for biocatalytic deacylation of A40926 (selective cleaving a fatty acid side chain of the molecule (R1 in Fig.1)) to obtain the desired deacyl-A40926 derivative (Chapter 2). This biotransformation is carried out with whole resting cells. Because we were developing a continuous process with non-growing cells, catalyst retention in the reactor was required. This is achieved by immobilizing cells in a Ca-alginate gel as a solid support (Chapter 2). Immobilized whole cell biocatalysis is most efficient when it is used in a continuous fashion, maximizing utilization of the catalyst. In a single lab-scale air-lift reactor continuous deacylation was accomplished (Chapter 4). We demonstrated that the process could stably operate for 3 weeks with an overall conversion of about 60%. Nonetheless, two important constraints necessitated overall bioconversion to be greater than 99%: 1) the very high costs of the substrate (A40926 itself is a natural antibiotic isolated from the fermentation broths through a complex recovery process), and 2) the very high purity of the final product needed (deacyl-A40926 is subsequently used for selective reacylation to generate novel derivatives). The solution to this was finally found in a series of three perfectly mixed air-lift bioreactors (Chapter 5). A continuously operated cascade system was run for almost two months with an overall conversion of >99%, demonstrating the feasibility of the continuous deacylation at the lab-scale. With this process configuration we were able to produce gram quantities of deacyl-A40926.

As discussed above, deacyl-A40926 is an ideal scaffold that can be selectively reacylated with different acyl chains or functional groups to generate novel and more potent compounds. It is also an indispensable element for structure-activity relationships (SAR) studies, an essential part of a continuous search for better glycopeptides. Further on in this chapter we discuss this never ending battle against multi-resistant bacteria together with

some recent developments and possible alternatives in quest of better drugs and /or treatments against MRSA.

It all begun with penicillins

The use of penicillin as an antibiotic is one of the most important breakthroughs in human health care. Although penicillin was discovered by Fleming in 1928, its application was not realized until Chain and Florey contributed their efforts about a decade later¹. Fleming, Chain and Florey won the Nobel Prize in 1945 for the discovery of penicillin and its curative effect in infectious diseases². Since then, penicillin and many of its derivatives have become, and still are, the first-line medicine to treat bacterial infections. The development of large-scale production processes for penicillins has strongly stimulated the development of modern biotechnology. Most antibiotics prescribed today are derivatives of the ‘natural’ antibiotics produced by micro-organisms. A prominent example is the broad-spectrum semi-synthetic antibiotic and blockbuster amoxicillin that is derived from penicillin. Although most antibiotics used today were first employed 25 years or so ago, their manufacturing processes are still subject to significant improvement (Box 1). An elegant example is the protein evolution of a glutaryl acylase to produce a catalyst for the single-step (instead of two-step) hydrolysis of cephalosporin C into 7-aminocephalosporic acid (7-ACA)³.

Box 1. The Chemferm-project.

In 2001 a large research project on the synthesis of β -lactam antibiotics (penicillins and cephalosporins) was ended and the results summarized in a book⁴. It started as collaboration between three Dutch companies, DSM, Gist-brocades and their joint-venture Chemferm, and six academic groups at four Dutch universities. During the project the number of companies involved was reduced to one by the merger of DSM and Gist-brocades. The project was co-financed by the Dutch Ministry of Economic Affairs. A multi-disciplinary team of experts studied the scope and limitations of biocatalytic routes to the industrially most relevant penicillins and cephalosporins, as schematized in the general production chart in Figure 2. The results of the five-year intensive cooperation were published in over 100 scientific papers and several patents, representing guidelines for the

technological future of DSM Anti Infectives within the context of its business development.

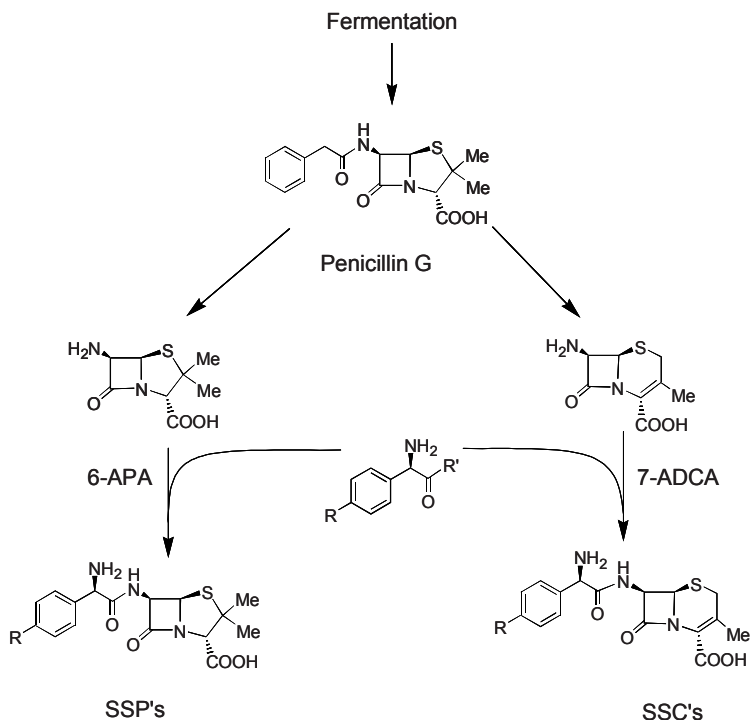


Figure 2 - General production chart of penicillin-derived antibiotics (semi-synthetic penicillins, SSP's, e.g. ampicillin (R = H) and amoxicillin (R = OH)) and cephalosporin-derived antibiotics (SSC's, e.g. cephalixin (R = H) and cephadroxil (R = OH)). Intermediates: 6-aminopenicillanic acid (6-APA) and 7-aminodesacetoxy-cephalosporanic acid (7-ADCA). Reproduced with permission from Tramper et al.⁵.

The occurrence of resistance

Only four years after pharmaceutical companies started manufacturing penicillin, bacteria began appearing that were resistant to it. *Staphylococcus aureus*, a Gram-positive bacterium that causes severe illnesses such as pneumonia and surgical-wound infections, could destroy penicillin by expressing the enzyme β -lactamase. In the late 1950's, penicillin was effective against only 15% of *Staphylococcus aureus* infections. The pharmaceutical industry responded to this emergence with methicillin, a β -lactamase-insensitive semi-synthetic penicillin. However, the first resistant clinical bacterial isolates

appeared in Europe during the 1960s, only a few years after the clinical introduction of methicillin. The crucial spread, however, started in the early 1980s in American hospitals. The resistant strain obtained the name methicillin-resistant *Staphylococcus aureus*, and later multi-resistant *Staphylococcus aureus* (MRSA), due to its resistance to against all β -lactam antibiotics.

Because bacterial evolution towards antibiotic resistance is considered a constant process the major effort of the health authorities and institutions is on delaying dissemination of resistant bacteria and/or resistance genes⁶. Resistance can result from mutations in resident structural or regulatory genes or from horizontal acquisition of foreign genetic information. Major resistance mechanisms can be distinguished:

1. Modification of the target that leads to loss or decreased affinity of the drug for its target, as for the mutated β -lactam-resistant transpeptidase.
2. Production of an enzyme that will detoxify the drug, such as β -lactamase.
3. Impermeability, in particular by reducing the number or the diameter of a porin (pore in the external membrane) in Gram-negative bacteria.
4. Efflux of antibiotics to the exterior of the bacterial cells by energy-dependent pumps, either in Gram-positive or Gram-negative pathogens.

The common objective of these various mechanisms is to impede interaction of the antibiotic with its target.

Antibiotics are classified according to their chemical structure, allowing division into four main classes: β -lactams, aminoglycosides, glycopeptides and macrolides. Members within a same class have similar molecular structures and generally the same antibiotic spectrum. They are, therefore, subject to cross-resistance by the two major ways that bacteria have developed:

1. By target alteration (mechanism 1 mentioned above), because the members share the same spectrum.
2. By making the members innocuous (mechanisms 2, 3 and 4 mentioned above), because they are structurally similar.

Thus, the resistance should be considered in terms of drug classes rather than in terms of isolated molecules⁶.

Alternatively to β -lactams, other antibacterials have been used against MRSA infections, e.g. tetracyclines, erythromycin, aminoglycosides and more recently the chemically derived fluoroquinolones. However, as a result of transposition and site-specific integrations of genetic information under the selective pressure exerted by the massive use and misuse of antibiotics, many MRSA have acquired resistance genes thus becoming insensitive to almost all known antibiotic classes. This situation where bacterial pathogens are evolving to become resistant to the full range of clinically useful antibiotics is a threatening scenario, almost equivalent to a return to the pre-antibiotic era.⁷ At present, the centre of concern is the antibiotic vancomycin that, for many infections, is literally the drug of 'last resort'. Some hospital-acquired *Staphylococcus aureus* infections are now resistant to all antibiotics except vancomycin⁸.

Unfortunately, vancomycin resistance has arisen in other common hospital bugs, i.e. enterococci, which are normally found in the human gut. The speed with which vancomycin resistance spread through enterococci prompted researchers to use the word 'crisis' when discussing the possibility of a vancomycin-resistant *Staphylococcus aureus* (VRSA)⁹. Indeed in 1997, signs of vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) were noted in patients hospitalized in three geographically different locations. Only a few years later, highly resistant VRSA were isolated following an event of *in vivo* horizontal resistant-gene transfer from *Enterococcus faecalis* to MRSA clinical isolates¹⁰. This alarm was taken seriously by all health authorities because vancomycin was thought the only weapon available against this strain. Clearly, resistance was indeed encroaching what is considered the last bastion of clinically effective antimicrobial agents, i.e. vancomycin and its relatives, the glycopeptide antibiotics¹¹. For a long time scientists and clinicians optimistically predicted that glycopeptide resistance would never occur because glycopeptides inhibit late steps in bacterial cell wall peptidoglycan synthesis, an antibiotic target difficult to modify for bacteria. However, as one of the most remarkable

resistance strategies, it has become apparent that bacteria can actually use multiple proteins encoded on mobile transposons to essentially re-engineer their peptidoglycan and prevent binding of the antibiotic vancomycin, while still retaining the structural integrity of their cell wall⁷.

Glycopeptide antibiotics

In 1956, scientists from Eli Lilly discovered vancomycin. They recovered this compound from the culture of the actinomycete *Streptomyces orientalis*, today classified as *Amycolatopsis orientalis*. The purified natural product exhibited lethal properties against all tested strains of *Staphylococcus* and other Gram-positive bacteria. The substance was coined vancomycin, the name being derived from the verb ‘to vanquish’. Vancomycin became available for clinical use upon its FDA approval in 1958. The introduction of vancomycin as an anti-staphylococcal agent was followed shortly thereafter by methicillin and cephalosporins, drugs that initially received wider clinical acceptance in contrast to vancomycin because of the apparent toxic side effects of the latter. Vancomycin popularity increased as its purity improved, which alleviated many side effects. Today, vancomycin and its sister antibiotic teicoplanin, the latter being discovered by researchers of the Lepetit group (Box 2) and introduced in clinics in Europe in 1988¹², are still indispensable weapons of the clinician facing life-threatening situations with patients infected with Gram-positive, drug-resistant bacterial strains.

Box 2. Vicissitudes of a typical anti-infectives biotech company.

Lepetit Research Centre was until 1995 a medium-sized (100-150 employees) research laboratory, belonging to Marion Merrell Dow, devoted to the discovery and development of novel anti-infectives. It was established in Gerezano near Milan, Italy. Lepetit discovered rifamycin followed by teicoplanin, ramoplanin, lantibiotic actagardine, A40926 and dalbavancin. In 1995 Lepetit was bought by Hoechst and turned into Hoechst Marion Roussel, representing the pharmaceutical branch of Hoechst. As result of this operation Biosearch Italia arose as a spin-off in 1997. Biosearch Italia presented itself as a small Italian biopharmaceutical company focusing on new antibiotics for the treatment of infections caused by multi-resistant bacteria. They specialized in glycopeptides, the class

of antibiotics to which vancomycin, teicoplanin, A40926 and dalbavancin belong. At that time the company worked together with Wageningen University, the Netherlands, on the de-acylation of A40926 (Figure 3) with immobilized cells in airlift bioreactors. In 2000 Biosearch Italia became the first small biotech company in Italy that went public and appeared on the Nuovo Mercato stock exchange. Then in 2003 it merged with the American biopharmaceutical company Fremont-based Versicor Inc. into Vicuron Pharmaceuticals (listed on both the NASDAQ and Nuovo Mercato stock exchange). At that time the company had three molecules in the clinical pipeline (Phase II and III), i.e. dalbavancin, anidulafungin and ramoplanin. In 2005 the company was bought by Pfizer for \$ 1,9 billion who brought anidulafungin on the market under the trade name Eraxis. In 2006 Pfizer decided to restructure its R&D and closed the research centre in Gerezano. Since 2007 three of the former scientists go on as Actygea S.r.l., a starting biotech enterprise that focuses on R&D in the field of microbial products.

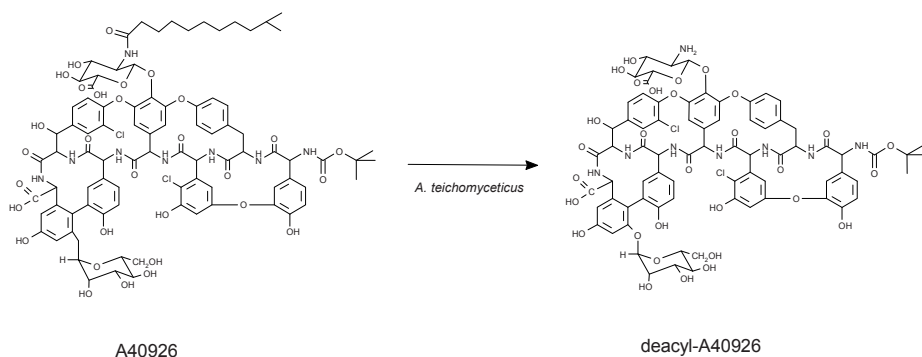


Figure 3. Biotransformation of A40926 *t*-BOC derivative to deacyl-A40926 *t*-BOC derivative by *Actinoplanes teichomyceticus*¹³.

Before 1984, the glycopeptide class included few members beyond vancomycin and teicoplanin. With the threat of resistance, the class has rapidly grown since then. Hundreds of related natural products have been discovered meanwhile and thousands of semi-synthetic analogues have been prepared. These constitute the class of compounds having a core heptapeptide scaffold containing aromatic amino acids that have undergone extensive oxidative cross-linking and decoration with different substituents such as sugar residues, chlorine atoms and/or lipid chains. Structural studies on these compounds have clarified the biological mode of action and serve as a basis for reasonable predictions regarding

structure–activity relationships (SARs). Early attempts to elucidate the structure of vancomycin were hampered by impurities, lack of crystallinity, and structural complexity. With improvements in purification methods and development of newer spectroscopic techniques, pioneering studies on the structure of vancomycin became possible and, in 1982, the complete structure was elucidated. Several other glycopeptides followed. In 1995, the first crystallographic analysis of an intact, naturally occurring glycopeptide antibiotic, balhimycin, was reported, and one year later the crystal structure of vancomycin was also established.

As their name suggests, the glycopeptide antibiotics normally contain sugar moieties. A wide variety of carbohydrates have been found to occupy positions on the peptide backbone. These carbohydrate units are attached to the aglycon (the basic structure without sugar residues) through glycosidic bonds to phenolic or secondary hydroxy groups. The sugar groups of the glycopeptides play important roles in delivering the antibiotic to its target by modulating its solubility. The sugar domains of these molecules often promote a stronger binding and improved *in vivo* activity. The glycopeptide antibiotics exhibit various degrees of glycosidation, ranging from displaying no sugar residues, e.g. the vancomycin and teicoplanin aglycon, to carrying several sugar units with up to four glycosidic bonds linking them to the heptapeptide core, i.e. the highly adorned galacardin A (two disaccharides and two monosaccharides directly linked to the heptapeptide core. Box 3 describes the mode of action of glycopeptides.

Box 3 Mode of action of glycopeptides

Glycopeptides are active against Gram-positive pathogens. They act by inhibiting the extracellular assembly of cell wall peptidoglycan. Binding of vancomycin to the bacterial cell wall was known long before its structural elucidation. Today, a precise structural picture for this interaction has been uncovered by NMR and X-ray crystallographic techniques. It is now known that vancomycin binds reversibly to the D-Ala-D-Ala fragment of a peptidoglycan precursor. This reversible, non-covalent interaction by five well-defined hydrogen bonds inhibits transglycosylation and transpeptidation from occurring (Figure 4). Inhibition of these processes leads to the collapse of the peptidoglycan by

decisively shifting its dynamic equilibrium towards de-assembly, which causes cell lysis and bacterial death. Further to this, a number of secondary effects of glycopeptide antibiotics enhance their potency. Prominent among these secondary effects are the abilities of a number of glycopeptides to dimerize in solution and of others to anchor themselves into the phospholipid bilayer by using lipophilic tails. NMR studies and X-ray crystallographic data show that vancomycin and a number of other glycopeptides could dimerize in solution to form 'head-to-tail' complexes. The dimeric structures, held together by four hydrogen bonds, contain two binding sites for L-Lys-D-Ala-D-Ala units. The tendency of glycopeptide antibiotics to dimerize has been correlated with their potency. Two hypothesis are currently used to explain this phenomenon. The first predicts a higher activity for a dimer because once half of the dimer binds to the substrate as usual, the other half then finds its target through what now becomes essentially intra-molecular binding.

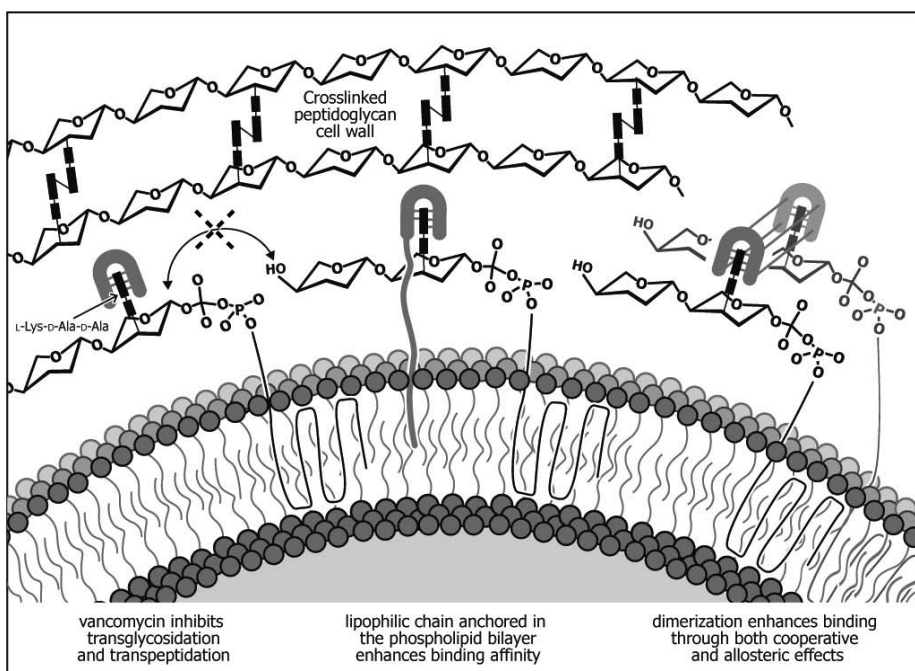


Figure 4 - Mechanisms of action of glycopeptides antibiotics (adapted from Nicolaou et al.¹¹).

This cooperative effect decreases the entropy factor for binding. The second hypothesis predicts increased activity through allosteric effects. Thus, the hydrogen bonding within the dimer enhances the ability of the binding pocket to bind the ligand by polarizing the amide bonds. This effect works in reverse as well. That is to say, the binding of the ligand also enhances the ability of the glycopeptide

antibiotic to dimerize. In contrast to vancomycin, teicoplanin exhibits high antibiotic activity despite its inability to dimerize in solution. An explanation for the enhanced potency of teicoplanin is provided by another secondary effect exhibited by a number of glycopeptide antibiotics, that of anchoring into the cell's phospholipid bilayer through a long hydrocarbon chain. Thus, the lipophilic tail attached to one of its carbohydrate units of teicoplanin localizes the antibiotic to its site of action. To expand the antibiotic spectrum to Gram-negative bacteria chemists at the former Lepetit Laboratories (Box 2) prepared mideplanin by 3-(N,N-dimethylamino) propylamidation of teicoplanin. This derivative, and the one similarly derived from the teicoplanin aglycon, have some activity against both staphylococci and Gram-negative bacteria. Furthermore, two teicoplanin aglycon-derived poly-(aminopropyl)amides also exhibited significant antibiotic activity against Gram-negative bacteria. These modified glycopeptides do not differ from their parent compounds except for their ability to traverse the outer cell membrane of the Gram-negative bacteria by a self-promoted uptake mechanism¹⁴.

Resistance build-up

After several decades of use, appearance of the resistance to glycopeptides in enterococci, followed by its transfer to methicillin-resistant *Staphylococcus aureus*, came rather as a surprise⁶. Drastic alterations in the synthesis of the peptidoglycan must have occurred to prevent binding with the antibiotic, and such changes would involve multiple mutations. Alteration of the target of glycopeptides by replacing the D-Ala-D-Ala terminus of the peptidoglycan precursor with the depsipeptide D-Ala-D-Lac reduces the antibiotic affinity by three orders of magnitude. This was not the result of spontaneous mutations in the clinical isolate, but it came from events of horizontal gene transfers from the glycopeptide-producing microorganisms, where the set of *van* genes (so called since they confer vancomycin resistance) are useful to avoid self-inhibition during antibiotic production. Not only have enterococci acquired these genes that allow remodeling of the synthesis of the bacterial cell wall, but they did so in respecting the principle of parsimony. Acquired resistance to glycopeptides provides an interesting example of obligatory cooperation of resistance mechanisms, namely synthesis of a new 'insensitive' target (D-Ala-D-Lac) combined with removal of the previous 'susceptible' target (D-Ala-D-Ala). In fact the cluster of *van* genes comprises enzymes able to produce the D-Ala-D-Lac depsipeptide, but

also enzymes to remove the preformed D-Ala-D-Ala. As long as bacteria synthesize wild-type precursors (D-Ala-D-Ala), they remain susceptible to the drugs. There is therefore a need to eliminate the normal precursors as the removal of the susceptible target lead to dominance of resistance versus susceptibility. This sophisticated resistance mechanism is presumably energetically extremely costly for the cell, which builds and then destroys cell wall precursors. Therefore activation of the resistance pathway and simultaneous deactivation of the chromosomal pathway should only occur when this switch in metabolic pathways confers a selective advantage to the host. It is therefore logical that the presence of sub-inhibitory concentrations of glycopeptides in the culture medium triggers expression of resistance. Although glycopeptide resistance was predicted not to occur, it could have been anticipated by the study of the producing microorganisms⁶. Indeed, *van* genes encoding homologues of the resistance proteins in enterococci have been identified in the strains of *Amycolatopsis orientalis*, *Actinoplanes teichomyceticus* and *Streptomyces toyocaensis* that are used for the industrial production of vancomycin, teicoplanin and the sulfated teicoplanin-like aglycone A47934, respectively^{15, 16}.

Perspectives

As we enter the 21st century we are faced with an ever-increasing need for a new generation of antibiotics capable of fighting the deadly, drug-resistant bacteria²⁸. The urgency is aggravated even more, because of an almost empty clinical pipeline of new antibiotics. A remarkable innovation gap of almost 40 years that was witnessed, between the introduction of quinolones in 1962 and the approval of the next new structural class of antibiotic, the oxazolidinone linezolid (Zyvox), is a warning sign for the future. Inspecting the antibacterial drug candidates that are being advanced through clinical trials, it can be seen that the innovation gap still exists as many drug candidates under development continue to be minor modifications of the existing scaffolds. Another alarming indicator of the almost empty antibiotic clinical pipeline is the fact that few large pharmaceutical companies remain active in antibiotic development. The exit and/or de-emphasis of many pharmaceutical firms from this therapeutic area reflect not only a mix of economic and market projections, but also a partial to complete failure of research programs that have

been built on existing models to find new leads that are robust enough to become clinical candidates¹⁷. As a consequence, we witnessed in the last decade a shift in emphasis of anti-bacterial R&D efforts away from many large pharmaceutical firms to a large contingent of biotech companies. This shift, combined with the entrepreneur approach to discovery, has led to a new wave of creativity in strategies, selection of targets, genomics, and development paradigms, but with scarce success.¹⁸ Following the publication in 1995 of the first whole-genome sequences of two bacterial pathogens – *Haemophilus influenzae* and *Mycoplasma genitalium* – both academic and industrial laboratories launched a wave of ‘genomics’ efforts towards the identification of novel bacterial targets. The effort was typically an outstanding scientific accomplishment – usually paired with a DNA-sequence-based genomic patent position on certain unique or proprietary targets – but no quality drug candidate emerged from these efforts¹⁹. Striking example is a GlaxoSmithKline (GSK) campaign (1995-2001) to discover broad-spectrum antibiotics by the genomic approach. With the tools to search entire genomes for new antibacterial targets at hand, GSK evaluated more than 300 genes in 70 high-throughput campaigns over 7 years, but ultimately failed because of the limited chemical diversity of the synthetic screening libraries²². It is therefore safe to say that a good established target is better than a new target. Thus, a novel strategies should consist of expanding the structural diversity and screening new compounds against well-established targets. This, in part, has already been happening in recent years through resurgence of the discovery of natural product antibiotics from bacterial sources. As technology advanced, efforts have refocused either on finding new antibiotics from old sources (like streptomycetes) or from new sources (like other actinomycetes, cyanobacteria and uncultured bacteria)²⁰, or on rediscovering old ones by semi-synthetic tailoring of natural product scaffolds. Focusing on those antibacterials that act by inhibiting cell wall biosynthesis, which still remains a well-established and robust target for natural product screenings, new or newly rediscovered antibiotics like oritavancin, telavancin and dalbavancin (a semi-synthetic derivative of A40926) were brought to a clinical development²⁷. Very good examples of newly rediscovered compounds are mannopeptimycins, ramoplanin and lantibiotics. All of them inhibit some steps of the Lipid II cycle or block the flux of peptidoglycan precursors to the cross-linking

transglycosidases and transpeptidases (as outlined in Box 4).

Box 4 The mannopeptimycins

The mannopeptimycins are a family of glycopeptides first isolated from Streptomyces species in the 1950s. They inhibit Lipid II-dependent peptidoglycan maturation by binding to Lipid II in a way that is different from other known compounds (Figure 5).

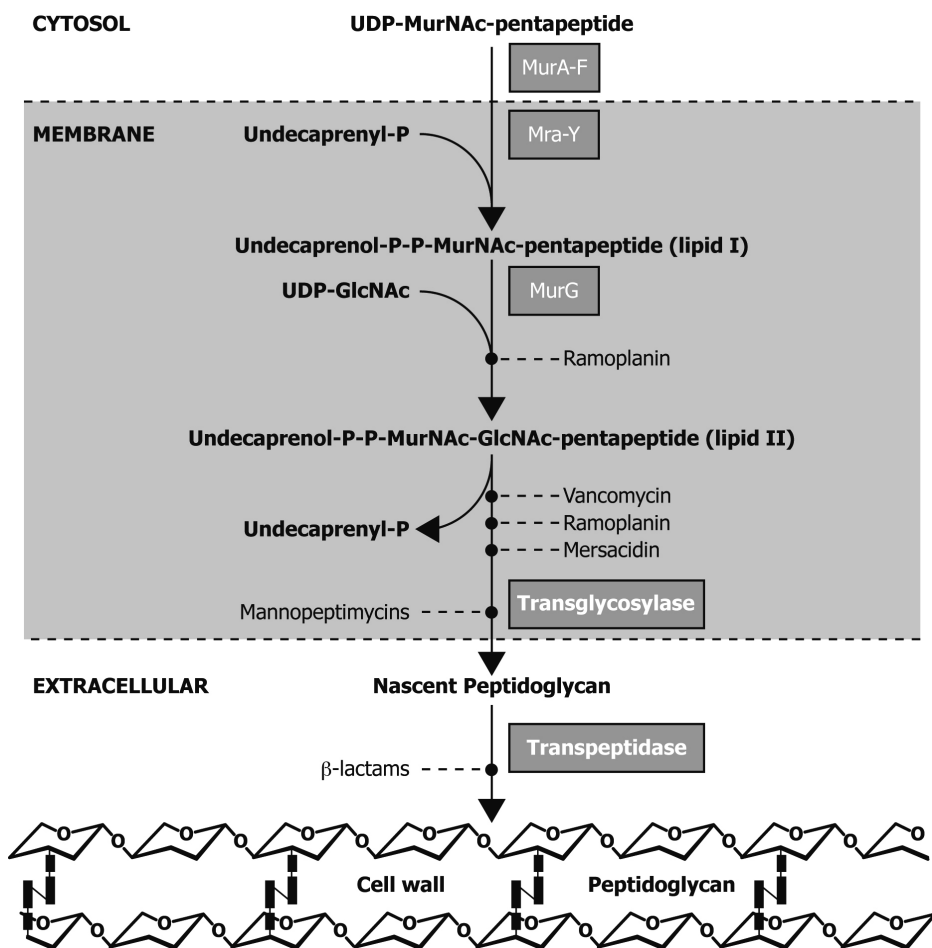


Figure 5 - Lipid II pathway and site of action of mannopeptimycins and other lipid II inhibiting agents (adapted from Koehn²¹).

When mannopeptimycins were first discovered, they showed potent activity against Gram-positive organisms, but the chemical complexity of these compounds coupled with the lack of broad-spectrum activity reduced the prospects to further develop these antibiotics. The evolving clinical picture of bacterial resistance has today altered the profile of what are desirable antibiotics and creates opportunities for new strategies to discover them. Advances in HPLC, NMR, and mass spectrometry make it possible to isolate and structurally characterize what were previously intractable molecules. A semi-synthetic analogue program that sought to improve potency and in vivo activity resulted in a solid SAR picture and furnished numerous mannopeptimycin analogs with increased potency and serum stability²¹.

While antibiotics are still our last line of the defense, it is evident that there is the critical need for novel effective therapies against bacterial infectious diseases. Such alternative therapies should ideally put no selective pressure toward insurgence of the resistance among the bacteria. Interesting advancements in this direction could be found in recent developments of combination drugs (Box 5), or phage therapies²⁶, strategies that might become our additional weapons against multi-resistant bacteria^{23, 24}. Another emerging and very exciting resource for the discovery of new therapeutics are marine bacteria. They are considered to be a source with tremendous potential and if properly developed could provide the drugs needed to sustain our battle against drug-resistant infectious diseases in decades to come²⁹.

Box 5. Combination drugs, an emerging option for antibacterial therapy²³.

Combination of different classes of antibiotics or addition of adjuvants is a promising alternative therapeutic approach (Figure 6). Combination of known and effective antibiotics from the various classes of antibiotics can not only enhance the antibacterial activity, but can possibly also support the clinical development of agents previously found to be very effective but too toxic for the host. Another advantage is that this approach might lead to shorter and/or lower dosing regimens, which has the potential to reduce the rate of acquirement of resistance in pathogens. A small number of combination therapies have already achieved commercial success for the treatment of resistant infections. In fact, the best-selling antibiotic in 2001 was the two-drug combination Augmentin®, marketed by GSK. It is a combination of the β -lactam antibiotic amoxicillin and an adjuvant, i.e. the lactamase inhibitor clavulanate. The β -lactam targets the cell wall whereas clavulanate prevents the

β -lactamase enzyme from degrading amoxicillin (mechanism 1). Bacteria become readily resistant against β -lactams as the gene coding for the lactamase is abundant and easily transferred from one bacterial strain to another.

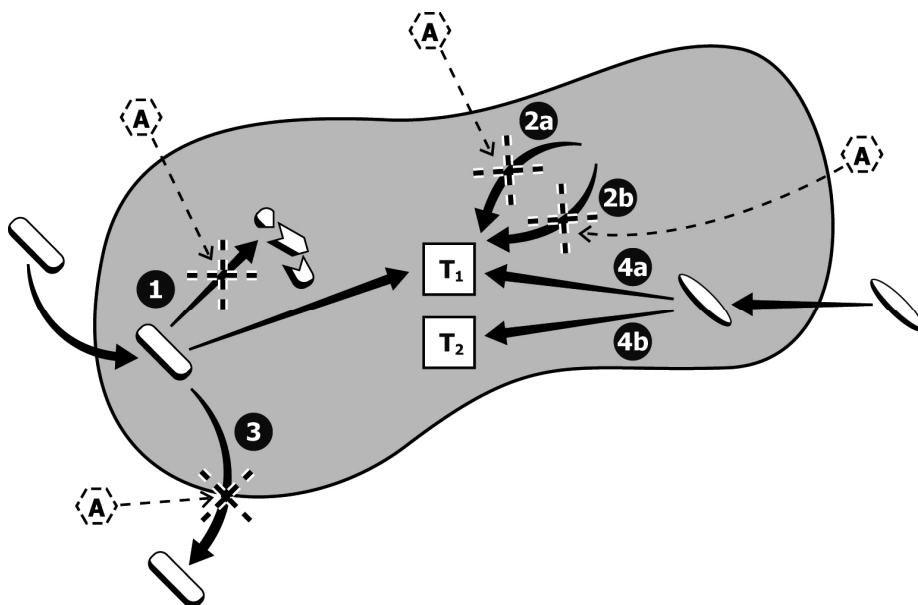


Figure 6. - Mechanisms of combination therapy: (1) Adjuvant (A) inhibits the degradation or modification of the drug; (2) adjuvant inhibits the cell repair (a) or intrinsic resistance pathway (b); (3) adjuvant inhibits the efflux pumps; (4) combination of two antibiotics with (a) or without (b) similar target T.

In addition to the synergistic effect on drug efficacy, combination therapy has the potential to slow the emergence of resistance. The underlying hypothesis is that treatments that inhibit multiple targets (on the same pathway or not) might delay and decrease the pathogen's ability to accumulate simultaneous mutations that affect the multiple targets. This concept has been well documented for tuberculosis, malaria and leprosy.

Conclusions

New antibiotics are badly needed to combat the growing threat of bacterial resistance. Despite of significant advances in bacterial genomics, high-throughput screening techniques and synthetic methods, discovery of novel antibiotics over the past thirty years has not sufficiently kept pace with the demand for new agents. Fortunately, in the recent years there is ample evidence that microbial natural products continue to be a rich source of new drugs leads. This thesis demonstrates how natural products are and could be used in a quest of better drugs against multi-resistant bacteria.

As our battle with resistance is destined to continue, it is of utmost importance that we learn to use antibiotics cautiously and when appropriate. Only in this way we can delay dissemination of resistance, a natural phenomenon that will surely not disappear²⁵

References

1. Landsberg, H. (1949) Prelude to the Discovery of Penicillin. *Isis* 40, 225-227
2. Waksman, S.A. (1947) What Is an Antibiotic or an Antibiotic Substance? *Mycologia* 39, 565-569
3. Pollegioni, L., *et al.* (2005) Evolution of an acylase active on cephalosporin C. *Protein Science* 14, 3064-3076
4. Bruggink, A. (2001) Synthesis of Beta-Lactam Antibiotics: Chemistry, Biocatalysis & Process Integration. Kluwer Academic Publishers
5. Tramper, J., *et al.* (2001) Biocatalytic production of semi-synthetic cephalosporins: process technology and integration. In *Synthesis of β -lactam antibiotics - Chemistry, Biocatalysis and Process Integration*. (Bruggink, A., ed), 207, Kluwer Academic Publishers
6. Courvalin, P. (2008) Predictable and unpredictable evolution of antibiotic resistance. *Journal of Internal Medicine* 264, 4-16
7. Salmond, G.P.C., and Welch, M. (2008) Antibiotic resistance: adaptive evolution. *Lancet*, S97-S103
8. Lowy, F.D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. *Journal of Clinical Investigation* 111, 1265-1273
9. Larkin, M. (2003) Antibacterial resistance deemed a public-health crisis. *The Lancet Infectious Diseases* 3, 322-322
10. Appelbaum, P.C. (2006) The emergence of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 12, 16-23
11. Nicolaou, K.C., *et al.* (1999) Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew. Chem.-Int. Edit.* 38, 2096-2152
12. Parenti, F., *et al.* (2000) Teicoplanin Chemistry and Microbiology. *Journal of Chemotherapy* 12, 5-14
13. Jovetić, S., *et al.* (2006) Continuous biotransformation of glycopeptide antibiotic A40926 in a cascade of three airlift bioreactors using immobilized *Actinoplanes teichomyceticus* cells. *Enzyme Microb. Technol.* 38, 34-39
14. Malabarba, A., *et al.* (1997) Structural modifications of glycopeptide antibiotics. *Medicinal Research Reviews* 17, 69-137
15. Beltrametti, F., *et al.* (2007) Resistance to glycopeptide antibiotics in the teicoplanin producer is mediated by van gene homologue expression directing the synthesis of a modified cell wall peptidoglycan. *Antimicrob. Agents Chemother.* 51, 1135-1141

16. Marshall, C.G., *et al.* (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob. Agents Chemother.* 42, 2215-2220
17. Walsh, C. (2003) Where will new antibiotics come from? *Nat. Rev. Microbiol.* 1, 65-70
18. Overbye, K.M., and Barrett, J.F. (2005) Antibiotics: where did we go wrong. *Drug Discov. Today* 10, 45-52
19. Barrett, J.F. (2005) Can biotech deliver new antibiotics? *Curr. Opin. Microbiol.* 8, 498-503
20. Clardy, J., *et al.* (2006) New antibiotics from bacterial natural products. *Nat. Biotechnol.* 24, 1541-1550
21. Koehn, F.E. (2008) New strategies and methods in the discovery of natural product anti-infective agents: The mannopeptimycins. *Journal of Medicinal Chemistry* 51, 2613-2617
22. Payne, D.J., *et al.* (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discov.* 6, 29-40
23. Cottarel, G., and Wierzbowski, J. (2007) Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol.* 25, 547-555
24. Payne, D.J. (2008) Microbiology - Desperately seeking new antibiotics. *Science* 321, 1644-1645
25. Martinez, J.L., *et al.* (2009) A global view of antibiotic resistance. *Fems Microbiol. Rev.* 33, 44-65
26. Sulakvelidze, A. 2005. Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. *Drug Discovery Today* **10** (12), 807-809.
27. Van Bambeke, F. 2004. Glycopeptides in clinical development: pharmacological profile and clinical perspectives. *Current Opinion in Pharmacology* **4** (5), 471-478.
28. Abbanat, D., Morrow, B., Bush, K. 2008. New agents in development for the treatment of bacterial infections. *Current Opinion in Pharmacology* **8** (5), 582-592.
29. Williams, P.G. 2009. Panning for chemical gold: marine bacteria as a source of new therapeutics. *Trends in Biotechnology* **27** (1), 45-52.

Summary

Glycopeptides are fascinating molecules with a strong inhibitory activity against Gram-positive bacteria. The two best known and today still the only glycopeptide antibiotics commercially available for human use are vancomycin and teicoplanin. They are often referred to as drugs of last resort against serious hospital infections caused by multidrug-resistant Gram-positive pathogens, particularly methicillin-resistant *Staphylococcus aureus* (MRSA).

A40926 is a prominent member of the glycopeptide antibiotic family and structurally related to teicoplanin. It is commercially a very important molecule as it is the precursor for the semi-synthesis of dalbavancin, a novel second-generation glycopeptide agent currently in a late stage of the clinical development for the treatment of patients infected with the most difficult-to-treat strains of MRSA.

The multifaceted structure of A40926 makes it an ideal and at the same time challenging scaffold for derivatization. This, combined with an excellent activity against Gram-positive *Staphylococcus aureus* offers a unique opportunity for the discovery and the development of new semisynthetic glycopeptide antibiotics.

In the work describe in this thesis the selective deacylation of A40926 by immobilized cells of *Actynoplanes teichomyceticus* was investigated. By selective cleaving a fatty acid side chain of the molecule, a deacyl-A40926 derivative is obtained. This derivative can than selectively be reacylated with different acyl chains or functional groups to generate novel compounds. Above that, deacyl-A40926 is an indispensable element in structure-activity relationships (SAR) studies that are at the core of any drug development program.

The main objective of this thesis research was to develop a lab-scale prototype process for continuous deacylation of A40926 that could produce gram quantities of deacyl-A40926, subsequently to be used as a precursor for chemical derivatization. This was achieved by implementing a continuously operated cascade of three perfectly mixed airlift loop reactors

(ALR) with immobilized *A. teichomyceticus* cells. For the design purposes effective diffusion coefficients of A40926 and its deacylated derivative were determined (Chapter 3) together with kinetic parameters (Chapter 4). That allowed us to model a process initially based on a single continuously operated air-lift reactor (Chapter 4). Subsequently, because very high overall conversion was required, a process based on a series of three ALRs was designed. This configuration was experimentally implemented at lab scale and operated continuously for two months (Chapter 5), giving a desired overall conversion of A40926 to deacyl-A40926 of more than 99%. Thus we demonstrated the feasibility of the continuous deacylation and were able to produce gram quantities of deacyl-A40926.

Chemical modification, such as deacylation, of complex glycopeptide molecules is an almost impossible task and the solution to this should be sought in the form of biocatalysis. This research is a demonstration that biotransformation and bioprocess development are an indispensable part of a much larger process of discovering and developing new drugs. New and better drugs that we will continue to need in our never-ending battle against multi-resistant bacterial infectious diseases.

Samenvatting

Glycopeptiden zijn fascinerende moleculen met een sterk remmende werking op gram-positieve bacteriën. De twee bekendste glycopeptideantibiotica – de enige antibiotica voor de mens die op dit moment commercieel verkrijgbaar zijn – zijn vancomycine en teicoplanine. Ze worden vaak gebruikt als een laatste redmiddel tegen ernstige ziekenhuisinfecties die worden veroorzaakt door multiresistente gram-positieve pathogenen, voornamelijk methicilline-resistente *Staphylococcus aureus* (MRSA).

A40926 is een bekend glycopeptideantibioticum, dat qua structuur aan teicoplanine gerelateerd is. Commercieel gezien is het een zeer belangrijk molecuul omdat het de voorloper is van de semi-synthese van dalbavancine, een nieuw tweede generatie glycopeptideantibioticum dat in een laat stadium van klinische ontwikkeling is, waarmee patiënten kunnen worden behandeld die met de slechtst behandelbare varianten van MRSA zijn besmet.

A40926 is met zijn veelzijdige structuur een ideale, maar tegelijkertijd lastige scaffold voor derivatisatie. In combinatie met een uitstekende werking tegen gram-positieve *Staphylococcus aureus* biedt dit een unieke kans om nieuwe semi-synthetische glycopeptideantibiotica te ontdekken en te ontwikkelen.

Het onderzoek dat in deze thesis wordt beschreven, was gericht op de selectieve deacylatie van A40926 door geïmmobiliseerde *Actynoplanes teichomyceticus*-cellen. Door een vetzuurzijketen van het molecuul selectief af te splitsen, wordt een deacyl-A40926-derivaat verkregen. Dit derivaat kan dan selectief opnieuw worden geacyleerd met verschillende acylketens of functionele groepen om nieuwe samenstellingen te genereren. Bovendien is deacyl-A40926 een onmisbaar element in onderzoeken naar de structuur-activiteitsrelatie, die van essentieel belang zijn voor het ontwikkelen van medicijnen.

Het hoofddoel van dit thesisonderzoek was om een prototype te ontwikkelen van een laboratoriumproces voor continue deacylatie van A40926 om gramhoeveelheden van

deacyl-A40926 te produceren, die vervolgens konden worden gebruikt als precursor voor chemische derivatisatie. Dit werd bereikt door het implementeren van drie continu werkende, perfect gemengde airlift loop reactors (ALR) met geïmmobiliseerde *A. teichomyceticus*-cellen. Eerst werden effectieve diffusiecoëfficiënten van A40926 en van het gedeacyleerde derivaat (hoofdstuk 3) en kinetische parameters (hoofdstuk 4) bepaald. Zo konden we een proces ontwikkelen dat in eerste instantie was gebaseerd op één continu werkende airlift reactor (hoofdstuk 4). Vervolgens werd er een proces op basis van drie ALR's uitgedacht, omdat er een zeer hoge overall-conversie vereist was. Deze continu werkende opstelling werd bij wijze van experiment gedurende twee maanden in een laboratorium geïmplementeerd (hoofdstuk 5). Dit leidde tot een gewenste overall-conversie van A40926 naar deacyl-A40926 van meer dan 99%. Daaruit blijkt dat continue deacylatie mogelijk is en dat we in staat waren om gramhoeveelheden van deacyl-A40926 te produceren.

Chemische modificatie, zoals deacylatie, van complexe glycopeptidemoleculen is een bijna onmogelijke taak en de oplossing moet worden gezocht in het gebruik van biokatalytische processen. Dit onderzoek toont aan dat biotransformatie en bioprocesontwikkeling onmisbaar zijn voor het zeer uitgebreide proces waarmee het ontdekken en ontwikkelen van nieuwe medicijnen gepaard gaat. Nieuwe en betere medicijnen die we voortdurend nodig zullen hebben in onze eindeloze strijd tegen multiresistente bacteriële infectieziekten.

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On the last published paper of this thesis I had a very pleasant and fruitful collaboration with prof. Giancarlo Lancini, a man who contributed to the discovery of many important antibiotics at Lepetit Research Center and who is the world's authority in the field of anti-infectives. His vast knowledge and brisk thinking were always quite stimulating. I will remember with the greatest pleasure our discussions at the lunch in *Mensa* and during coffee-brakes, ranging from science to ancient and modern history, from philosophy to politics.

Last but not least, I want to remember people that were not directly involved in this thesis research but were (and some still are) in and around ‘Proceskunde’ when I was there. People, who together with Hans make the 6th floor of ‘Biotechnion’ an extremely stimulating and pleasant environment for me. Renè, Kees, Joyce, Rik, Arjen, Karin, Dirk, Marian, Fred, you will always be a part of this memorable experience.

Finally, thinking about this thesis I want to say to myself in Serbian *‘Bolje ikad nego nikad’* (Better ever than never), but also in Italian - *Meglio tardi che mai*, acknowledging this beautiful language and beautiful country that over the years offered me many opportunities, and to some extent became part of me.

About the author

Srdjan Jovetić was born in Belgrade (Serbia) on 25th of August 1963. After completing his secondary education in 5th Belgrade Gymnasium he enrolled at University of Belgrade to study Food Technology. He graduated in 1989 with a thesis on wine making and in 1990 started his postgraduate studies at the same University. Contemporarily he started his professional career as a research associate at PKB INI – Agroekonomik, an R&D company in Belgrade. In 1990, under Scientific Cooperation Program between (at that time) Yugoslavia and The Netherlands, he was awarded a two-year postgraduate study grant from the Dutch government. With this grant he came to Wageningen in 1991 and enrolled to MSc course in Biotechnology at Wageningen University. He graduated in 1993 with a thesis on baculovirus expression vector system, a research carried out at Bioprocess Engineering Group. After obtaining his MSc degree he spent two years in a group of prof. Tramper as a research assistant working on various research projects. In 1995 he went to Italy as detached research associate of Wageningen University to work at MMD-Lepetit Research Center. The project he was involved in was in collaboration with Marion-Merrel-Dow and a part of this PhD thesis research was done in that period. In 1997 he moved definitely to Italy to continue his scientific career at Biosearch Italia S.p.A. and later on at Vicuron Pharmaceuticals Inc. After spending ten years in industrial R&D covering various positions, in 2007 he co-founded *Actygea*, a start-up biotech company engaged in strain improvement and fermentation technology development.

Bibliography

Jovetić, S. 1994. Natural pyrethrins and biotechnological alternatives. *Biotechnology and Development Monitor* 21, 12-13.

Jovetić, S. and de Gooijer, C.D. 1995. The Production of Pyrethrins by in vitro Systems, *Critical Reviews in Biotechnology*, 15 (2), 125-138.

Jovetić, S., Tramper, J., Marinelli, F. 1998. Biotransformation of the lypoglycopeptide antibiotic A40926 with immobilized cells of *Actinoplanes teichomyceticus* - In situ supply of nutrients. *Enzyme and Microbial Technology*, 22, 117-121.

Jovetić, S., Beeftink, H.H., Tramper, J., Marinelli, F. 2001. Diffusion of (de)acylated antibiotic A40926 in alginate and carrageenan beads with or without cells and/or soybean meal. *Enzyme and Microbial Technology*, 28, 510-514.

Butler, M.J., Bruheim, P., Jovetić, S., Marinelli, F., Postma, P., Bibb, M.J. 2002. Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans*. *Applied and Environmental Microbiology*, 68 (10), 4731-4739.

Jovetić, S., de Bresser, L., Tramper, J., Marinelli, F. 2003. Deacylation of antibiotic A40926 by immobilized *Actinoplanes teichomyceticus* in an internal-loop air-lift bioreactor. *Enzyme and Microbial Technology*, 32, 546-552

Beltrametti, F., Lazzarini, A., Brunati, C., Marazzi, A., Jovetić, S., Selva, E., Marinelli, F. 2003. Production and Characterization of monochlorinated and dechlorinated A40926 derivatives. *Journal of Antibiotics*, 56, 773-782

M.J. Butler, E. Takano, P. Bruheim, S. Jovetić, F. Marinelli, M.J. Bibb. 2003. Deletion of *scbA* enhances antibiotic production in *Streptomyces lividans* 132. *Applied Microbiology and Biotechnology*, 61:512-516

Beltrametti, F., Jovetić, S., Feroggio, M., Gastaldo, L., Selva, E., Marinelli, F. 2004 Valine influences production and complex composition of glycopeptide antibiotic A40926 in fermentation of *Nonomuraea* sp. ATCC 39727. *Journal of Antibiotics*, 57, 37-44.

Jovetić, S., Marinelli, F., Tramper, J.,. 2006. Continuous biotransformation of glycopeptide antibiotic A40926 in a cascade of three airlift bioreactors using immobilized *Actinoplanes teichomyceticus* cells. *Enzyme and Microbial Technology*, 38, 34-39.

Gandolfi, R., Jovetić, S., Marinelli, F., Molinari, F. 2007 Biotransformations of lipoglycopeptides to obtain novel antibiotics. *Journal of Antibiotics*, 60(4), 265-271.

Di Palo, S., Gandolfi, R., Jovetić, S., Marinelli, F., Romano, D., Molinari, F. 2007 A new bacterial mannosidase for selective modifications of ramoplanin and its derivatives. *Enzyme and Microbial Technology*, 41, 806-811

Jovetić, S., Feroggio, M., Marinelli, F., Lancini, G. 2008 Factors influencing cell fatty acid composition and A40926 antibiotic complex production in *Nonomuraea* sp. ATCC 39727. *J Ind Microbiol Biotechnol* 35, 1131–1138

Jovetić, S., Zhu, Y., Marinelli, F., Tramper, J. β -lactam and glycopeptides antibiotics – first and last line of defence? Submitted for publication.

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