ISSN 1860-6768 · BJIOAM 3 (2) 141–280 (2008) · Vol. 3 · February 2008

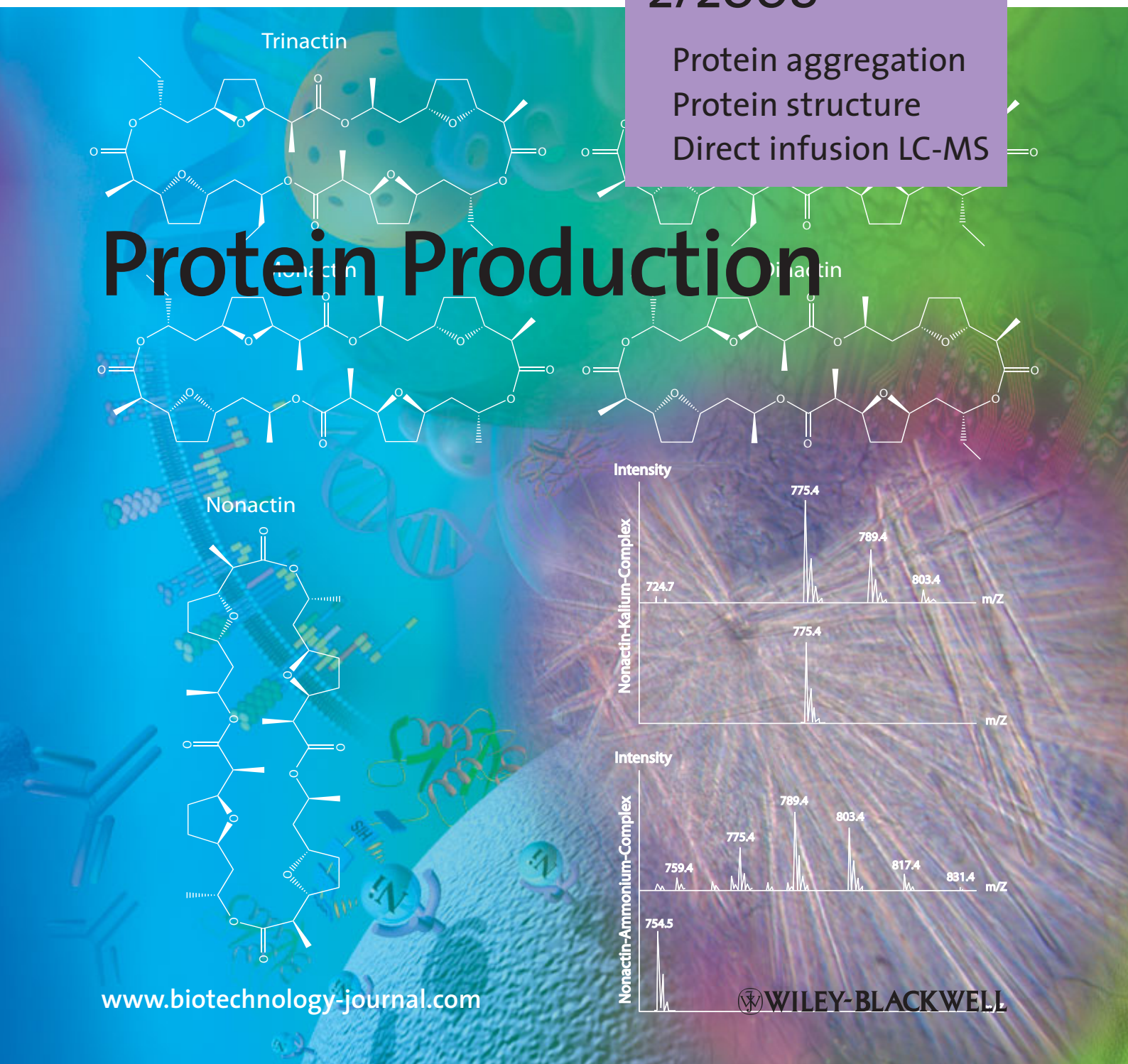
Biotechnology Journal

Healthcare · Nutrition · Biosafety

2/2008

Protein aggregation
Protein structure
Direct infusion LC-MS

Protein Production



www.biotechnology-journal.com

WILEY-BLACKWELL

DOI 10.1002/biot.200700174 Biotechnol. J. 2008, 3, 202–208

Research Article

Process analysis of macrotetrolide biosynthesis during fermentation by means of direct infusion LC-MS

Peter Jani, Joachim Emmert and Roland Wohlgemuth

Research Specialties, Sigma-Aldrich, Buchs, Switzerland

The optimization of the biosynthetic pathways is highly attractive for the large-scale preparation of macrotetrolides, because overall yields in the chemical synthesis of compounds like nonactin have been very low. A key success factor determining the outcome of such optimizations is the adequate process analysis for the envisioned product. The analytical methods for process control involved in the past spectrophotometric and chromatographic measurements. LC-MS offers a modern approach to obtain more detailed data than the spectrophotometric and chromatographic measurements used in the past. In this work, a fast and versatile analytical LC-MS method has been set up, which has proven of much value for the in-process analysis of macrotetrolides during fermentation and which has allowed rapid large-scale bioprocess development.

Received 5 August 2007
Revised 30 October 2007
Accepted 15 November 2007

Keywords: Mass spectrometry · Natural product biosynthesis · Nonactin/monactin/dinactin/trinactin/tetranactin · *Streptomyces griseus*

1 Introduction

Macrocyclization is an often-used molecular construction principle to obtain a certain limited bioactive conformational space of molecules with increased molecular recognition properties. The macrotetrolide nonactin and its homologues form a group of neutral lipophilic cyclic tetraesters with high biological activities [1], although the first and smallest homologue was given the name of nonactin, because it did not show any antibiotic activity worth mentioning [2]. After the first isolation in 1955, MS already was described as the most reliable method for the identification and purity determination of the homologues [3, 4]. The nonactin structure, initially deduced from spectroscopic analysis and later confirmed by X-ray analysis of the crystal structure, revealed an intriguing chirally alternating (+)-(–)-(+)-(–)-ester linkage of the enantiomeric nonactic acid-building blocks [5, 6], the other homologues of this family containing homononactic or bishomononactic acid as building blocks with the same relative configuration. As can be seen in Fig. 1, the macrotetrolide homologues nonactin, monactin, dinactin, trinactin and tetranactin are related to the macrocyclic backbone nonactin by the sequential substitution of one, two, three or four methyl groups by ethyl groups. Since nonactin is composed of two units of (+)-nonactate and two units of (–)-nonactate joined in such an alternating way that the whole molecule has S4 symmetry, it is achiral.

Macrotetrolide biosynthesis shows nature's versatility in making complex metabolites, and considerable biochemical and genetic work has been done to understand the biosynthetic pathway [7–14]. The unprecedented organization of a polyketide gene cluster reveals a novel type of polyketide synthase that features five genes that encode discrete type II ketoacylsynthase, four genes encoding discrete ketoreductase but lacks an acyl carrier protein. Of special interest is the proposal that biosynthesis diverges at the beta-ketoacyl reduction steps into a pair of enantiospecific pathways, each involving two ketoreductases [10].

Correspondence: Dr. Roland Wohlgemuth, Research Specialties, Sigma-Aldrich, Industriestrasse 25, CH-9470 Buchs, Switzerland
E-mail: rwohlgemuth@sial.com
Fax: +41-81-755-2584

Abbreviations: **EIC**, extracted ion current; **ESI**, electrospray interface

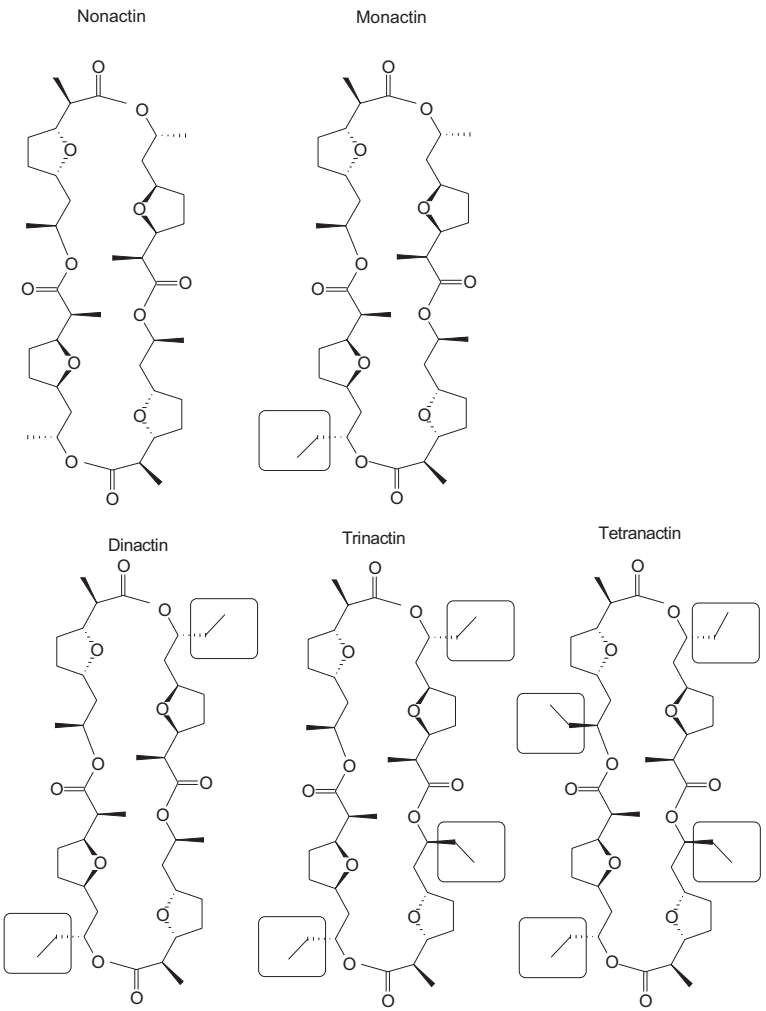


Figure 1. Macrotetrolide structures: nonactin, $C_{40}H_{64}O_{12}$, M=736.4; Monactin, $C_{41}H_{66}O_{12}$, M=750.5; dinactin, $C_{42}H_{68}O_{12}$, M=764.5; trinactin, $C_{43}H_{70}O_{12}$, M=778.5; tetranactin, $C_{44}H_{72}O_{12}$, M=792.5.

The chemical syntheses of macrotetrolides like nonactin have been achieved with stereocontrol over many steps, precise macrolactonization and building blocks of high enantio- and diastereomeric purity, but the overall yields have been very low [15–19]. It is therefore not surprising that from a preparative perspective the optimization of the biosynthetic pathway is highly attractive for the large-scale preparation of single-component and enantiomerically pure macrotetrolides. These high-purity macrotetrolides are used in sensors because of their molecular recognition properties for important cations [20].

The analytical methods for process control in the past involved spectrophotometric and chromatographic measurements with the bottlenecks of selectivity and sensitivity. LC-MS offers a modern approach to obtain data that are more detailed.

Positive LC-MS electrospray ionization normally yields the protonated molecular ion $[M+H]^+$, but some molecules exhibit a strong affinity to form alkali, mostly sodium adducts. Wang and Cole [21] investigated the influence of ionic strength on the positive-ion electrospray mass spectra of myoglobin and observed a decrease in signal intensity. The presence of alkali ions may be helpful in some cases [22, 23], especially when analyzing glycosides, or should be avoided [24], depending on the type of application. Cluster formation of alkali halogenides (*i.e.* CsI) can be used for calibration purposes or for MS quantitation of glycosidic drugs [25]. Schneider *et al.* [26] already described how polyether-ionophores could be measured at trace levels in MS by addition of different metal salts. Formation of metal-polyether ionophore-complexes was investigated by She and Brodbelt [27].

In the present work, a fast and versatile analytical LC-MS method has been developed for the in-process analysis of macrotetrolides during fermentation.

2 Materials and methods

2.1 Instrumentation, engineering, and reagents

Biotransformations were performed at 250-mL scale in mini-fermenters (Sixfors, Infors, Switzerland). The Sixfors multiple fermenter system is a bioreactor system that allows up to six separate fermentations with completely independent measurement and control of temperature, pH, stirrer speed and dissolved oxygen. The base unit provides the main electricity, water and gas supply as well as the holders for each vessel with electrical heating and cooling, magnetically coupled stirrer system eliminating the need for a mechanical seal system and leaving space above the holders for up to three peristaltic pump heads per fermenter station. The autoclavable fermenter vessels consist of borosilicate glass cylinders with a flat bottom and a stainless steel topplate with 2×12-mm port fittings closing the vessel by an easily releasable clamping system. The port fittings are designed to accept pH and oxygen electrodes, an exit gas cooler, gas sparger, entry points for reagent or nutrient feeds and a fixed Pt-100 temperature sensor. The mini-fermenters are fully equipped with feed pumps, temperature, oxygen, pH and anti-foam control. The pH-electrodes were tailor-made by Hamilton (Bonaduz, Switzerland). All six fermenters are controlled from a single industrial process controller that can be linked to a PC running the IRIS software for datalogging and control.

Peptone from soybeans, yeast extract, calcium carbonate, maltose, manganese sulfate, sodium nitrate, iron-(II)-sulfate heptahydrate, zinc sulfate heptahydrate, polypropyleneglycol, acetone and other chemicals were from Sigma-Aldrich (Buchs, Switzerland).

2.2 Fermentation and sample preparation

Precultures were grown at pH 7.4 in a medium consisting of 8 g/L peptone from soy-beans, 4 g/L yeast extract, 2 gL calcium carbonate and 15 g/L maltose (autoclaved separately to avoid Maillard reactions) for 3 days at 30°C and 180 rpm from lyophilisates of *Streptomyces griseus* Sg9 in shake flasks. For each Sixfors mini-fermenter position, 20-mL preculture that was checked by microscopic analysis for purity and mycel formation was used for inoculation, so that each reactor contained 250 mL total volume of a medium consisting of 4 g/L peptone from soybeans, 2 g/L yeast extract, 1 g/L calcium carbonate, 15 g/L maltose, 1 g/L polypropylenglycol, 0.3 g/L manganese sulfate, 1.5 g/L sodium nitrate, 0.15 g/L iron-(II)-sulfate heptahydrate and 0.025 g/L zinc sulfate heptahydrate. The Sixfors cultivation parameters were set at 30°C, 250 rpm, aeration 30 N L/h and pH was automatically regulated by the controlled addition of 2 M phosphoric acid or 2 M sodium hydroxide solution, respectively, at the different chosen values. The growth curves of *Streptomyces griseus* under different experimental conditions are shown in Fig. 2. Samples of 10 mL were regularly taken, centrifuged and extracted with 10 mL acetone for 2 h at 4°C and 1 mL of the supernatant used for analysis.

2.3 LC-MS analysis

The LC-MS measurements were performed with direct injection of standard or sample solution with

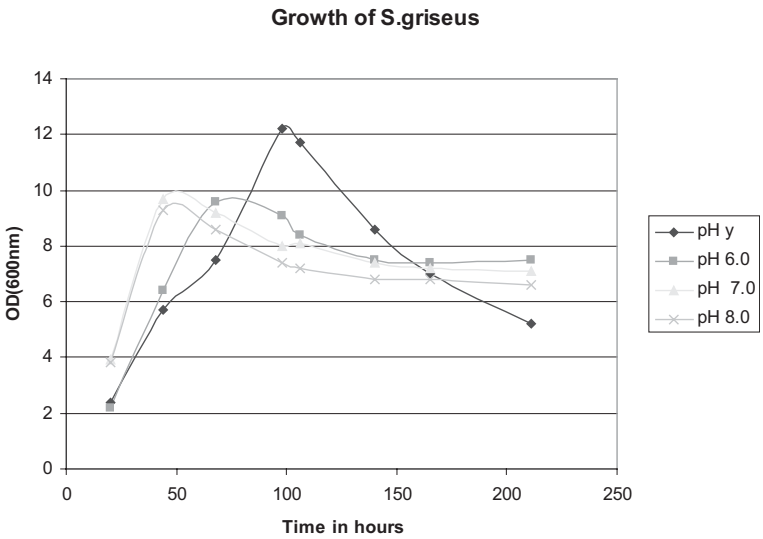


Figure 2. Growth of *Streptomyces griseus* as a function of pH.

a syringe pump (Cole Parmer, Vernon Hills, IL) at 4 µL/min into an ion trap mass spectrometer (Esquire 3000plus, Bruker Daltonics, Bremen, Germany). The mass spectrometer was equipped with an electrospray interface (ESI). The following reagents were used for the LC-MS analyses: nonactin (74155), ammonium acetate (09690), potassium acetate (00570), water (39253), water-isopropanol 50/50 v/v (34689) from Sigma-Aldrich (Buchs, Switzerland). Ammonium and potassium acetate were dissolved in water at a concentration of 0.1%.

Standard stock solution was prepared by dissolving nonactin standard in acetone at a concentration of 0.5 mg/mL. For MS measurements, this solution was diluted as follows: 100 µL stock solution, 100 µL potassium acetate 0.1% and 800 µL water-isopropanol for measurements as potassium adducts ("potassium mode") or 100 µL stock solution and 900 µL ammonium acetate 0.1% for measurements as ammonium adducts ("ammonium mode"). Of processed samples from fermentation, 100 µL was diluted with 900 µL potassium acetate 0.1% and measured in "potassium mode".

The electrospray parameters (temperature, capillary flow, nitrogen flow) were set to produce a stable spray and stream of ions, the mass spectrometer was operated in full-scan positive mode from m/z = 100–1000; all observed ions were singly charged. The parameter setting for the trap had to take into account the reduced stability of the K-adducts versus the pure macrotetrolides.

3 Results

In a first approach, spectrophotometric analysis of macrotetrolide formation yielded only global results and no detailed homologue concentrations. The production of pure macrotetrolide homologues requires however detailed insight into the biosynthesis of each homologue. Therefore, we have chosen LC-MS for process control.

As nonactin is an ammonium ionophore, the first experiment for LC-MS measurements was to use ammonium acetate as an additive to form the ammonium adduct $[M+NH_4]^+$, which has a mass of 754.5 DA (singly charged). This ("ammonium mode") worked well for a pure standard, but in process samples other ions present in the fermentation fluid interfered, resulting in very complex spectra. The series of macrotetrolides with mass differences of 14 (-CH_2-unit), was superimposed by a series of different ammonium and alkali adducts (NH_4, Na, K) of each macrotetrolide, resulting in a mass spectrum as shown in Fig. 3 (upper part). The spectrum in the lower part of the Fig. 3 is the theoretical spectrum of the nonactin ammonium adduct, while the corresponding results for the potassium adduct are shown in the upper and lower part of Fig. 4. Explanation of the signals is given in Table 1.

Obviously, the formation of the potassium adducts was more preferred in electrospray than with other alkali ions, so finally the measurement of process samples was only found to be consistent

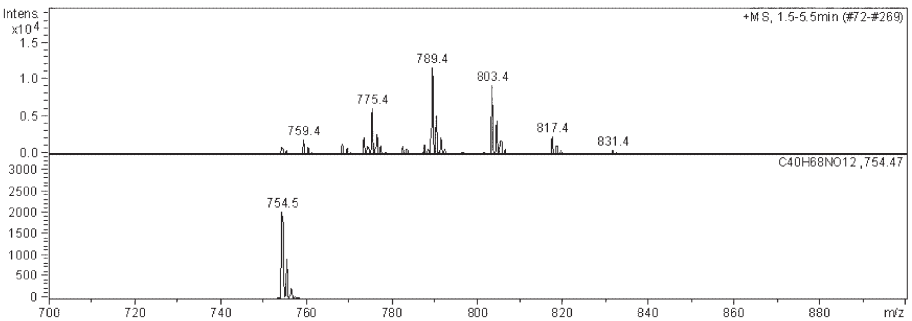


Figure 3. Mass spectrum of a process sample with addition of ammonium ions (upper part: real spectrum, lower part: theoretical spectrum).

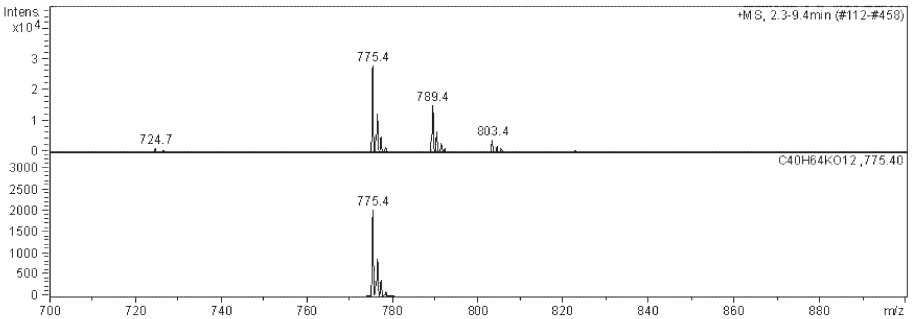


Figure 4. Mass spectrum of a process sample with addition of potassium ions (upper part: real spectrum, lower part: theoretical spectrum).

Biotechnol. J. 2008, 3, 202–208

Table 1. List of observed mass signals of process sample in NH_4-mode. In K-mode only the K adducts occur (**bold**)

M = 754.5	Nonactin + NH_4^+
M = 759.4	Nonactin + Na^+
M = 768.5	Monactin + NH_4^+
M = 773.4	Monactin + Na^+
M = 775.4	**Nonactin + K^+**
M = 782.5	Dinactin + NH_4^+
M = 787.5	Dinactin + Na^+
M = 789.4	**Monactin + K^+**
M = 803.4	**Dinactin + K^+**
M = 817.4	**Trinactin + K^+**
M = 831.4	**Tetranactin + K^+**

with an excess supply of potassium ions. These measurements in the so-called potassium mode showed the distribution of the macrotetrolides, all as potassium adducts $[M+K]^+$ which could be evaluated easily and observed during the fermentation process. Figures 5 and 6 show the time-dependent production of the macrotetrolide homologues during biosynthesis under different fermentation conditions. It can be seen, that they exhibit different maxima in terms of time and relative abundance, dependent on the pH of the fermentation medium. At pH 6, the biosynthesis of macrotetrolides is completely switched off, while pH 7 favors the formation of the homononactic acid-containing homologues with dinactin showing a maximum at 140 h, as can be seen in Fig. 5C. Monactin, trinactin and tetranactin are formed in smaller amounts but also having a peak at 140 h as can be seen in Figs. 5A and 6A and B. At pH 8, the same homononactic acid-containing homologues are formed reaching their maximum later at 160 h. The measurements of the homologue distribution by LC-MS represent a rapid and close to process analytical technology allowing understanding the influence of single parameters like pH, temperature and medium composition. The fermentation process could therefore be optimized adequately for production of a single molecular macrotetrolide entity.

Normally quantitation of compounds in LC-MS is done in chromatographic mode, using the extracted ion chromatogram (EIC) of the interesting mass. The area of the respective peak is integrated and calculated against a standard. As this was not possible here, when using spectral mode, the height of the monoisotopic mass of the different macrotetrolide-K-adducts was taken as a semiquantitative measure for their concentration. These intensity distributions of the different macrotetrolides were taken as a measure for their relative abundance during fermentation. The validity of this assumption was proven by a dilution experiment of a

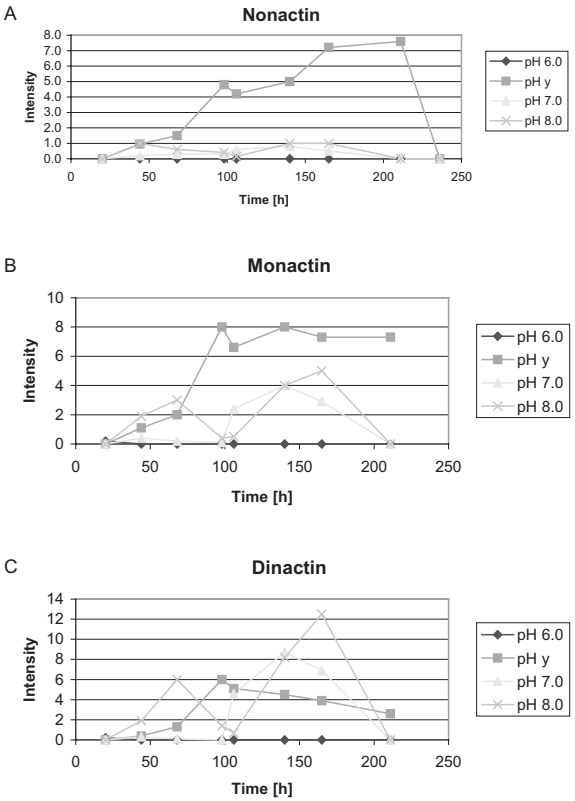


Figure 5. Biosynthesis of macrotetrolide homologues nonactin (A), monactin (B) and dinactin (C) as a function of pH and time; pH y means self-adjusted pH.

nonactin sample, containing about 8% monactin. The nonactin MS-signal intensities as a function of concentration are shown in Fig. 7. The stock solution was diluted 1:10, 1.20, 1:50 and 1:100, and the relative amount of the impurity monactin was calculated. Although the nonactin curve was not strictly linear, the amount of monactin was always calculated between 7.5 and 8.5%. The precision of this semiquantitative procedure was sufficient for the purpose of in-process control during the fermentation process.

4 Discussion

The quantitation of these macrotetrolide homologues by this MS method has the advantage that the bioprocess development can be downscaled and that smaller sample sizes are sufficient for quantification. This allows a more rapid process development compared to the traditional methods with immediate access to kinetic data. Colorimetric assays involving picric acid are also useful for rapid in-process control, but are not able to distinguish between different molecular entities of the

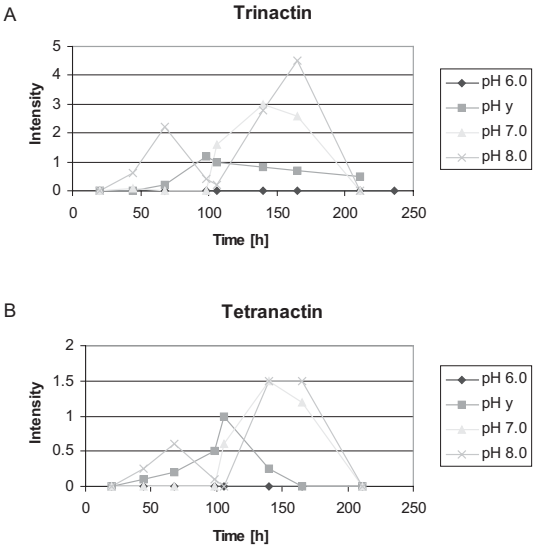


Figure 6. Biosynthesis of macrotetrolide homologues trinactin (6) and tetranactin (6) as a function of pH and time; pH y means self-adjusted pH.

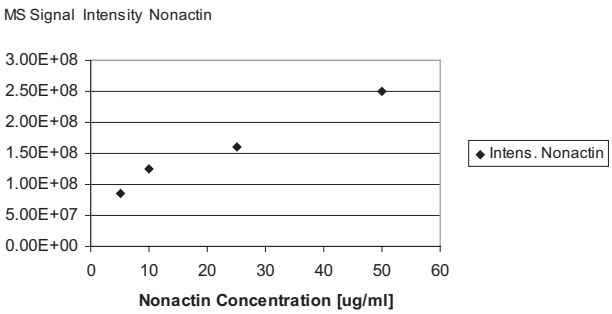


Figure 7. Dependence of nonactin MS-signal intensity on concentration (average values of 5-min direct infusion).

macrotetrolides. This nonspecific analysis can thus lead to development into the wrong direction for a specific homologue. In addition, these colorimetric assays are prone to influences from other components of the extracted medium. TLC and other chromatographic methods are time-consuming and suffer from the influence of medium components. The LC-MS method developed here overcomes all these disadvantages and provides an excellent tool for rapid and versatile determination of macrotetrolide homologues as a function of fermentation parameters like pH and time as illustrated in Figs. 5 and 6. In addition, medium optimization experiments for specific macrotetrolide formation, not just growth of *Streptomyces griseus*, are straightforward and transferable to fermenters of larger volumes. The selection of the medium components has also shown that the rapid analysis of the macrotetrolides is the key and that inorganic elements have a clear influence. The importance of calcium in the form of calcium carbonate or calcium nitrate in the medium has in this way been confirmed.

The method described above for the process analysis of macrotetrolides has not only been proven of enormous value in the achievement of robust large-scale manufacturing of nonactin, but also shows a great future in the process analysis of macrotetrolide homologues and other natural compounds showing process-dependent molecular diversity. This is becoming more important with the development of combined chemical and biological synthetic approaches like precursor-directed biosynthesis and mutational biosynthesis for the preparation of specific bioactive natural compounds [28].

In conclusion, we developed a highly sensitive and specific method, which simultaneously analyzes nonactin and related macrotetrolide homologues using direct infusion LC-MS in downscaled fermentation experiments.

The authors have declared no conflict of interest.

5 References

[1] Shen, B., Kwon, H.-J., Macrotetrolide biosynthesis: A novel type II polyketide synthase, *The Chemical Record* 2002, *2*, 389–396.

[2] Keller-Schierlein, W., Gerlach, H., Makrotetrolide, in: Zechmeister, L., (Ed.), *Fortschritte der Chemie organischer Naturstoffe XXVI*, Springer-Verlag New York, 1968, pp.161–189.

[3] Corbaz, R., Ettlinger, L., Gäumann, E., Keller-Schierlein, W. *et al.*, Stoffwechselprodukte von Actinomyceten. 3. Mitteilung, Nonactin, *Helv. Chim. Acta* 1955, *174*, 1445–1448.

[4] Gerlach, H., Hütter, R., Keller-Schierlein, W., Seibl, J., Zähner, H., Stoffwechselprodukte von Mikroorganismen 58. Mitteilung, Neue Makrotetrolide aus Actinomyceten, *Helv. Chim. Acta* 1967, *184*, 1782–1793.

[5] Dobler, M., The crystal structure of nonactin, *Helv. Chim. Acta* 1972, *55*, 1371–1384.

[6] Kilbourn, B. T., Dunitz, J. D., Ploda, L. A. R., Simon, W., Structure of the K^+ complex with nonactin, a macrotetrolide antibiotic possessing highly specific K^+ transport properties, *J. Mol. Biol.* 1967, *30*, 559–563.

[7] Woo, A. J., Strohl, W. R., Priestley, N. D., Nonactin biosynthesis: the product of *nonS* catalyzes the formation of the furan ring of nonactic acid, *Antimicrob. Agents Chemother.* 1999, *43*, 1662–1668.

[8] Smith, W. C., Xiang, L., Shen, B., Genetic localization and molecular characterization of the *nonS* gene required for macrotetrolide biosynthesis in *Streptomyces griseus* DSM40695, *Antimicrob. Agents Chemother.* 2000, *44*, 1809–1817.

[9] Ashworth, D. M., Clark, C. A., Robinson, J. A., On the biosynthetic origins of the hydrogen atoms in the macrotetrolide antibiotics and their mode of assembly catalyzed by a non-

actin polyketide synthase, *J. Chem. Soc. Perkin Trans. I* 1989, *8*, 1461–1467.

[10] Nelson, M. E., Priestley, N. D., Nonactin biosynthesis: The initial committed step is the condensation of acetate (malonate) and succinate, *J. Am. Chem. Soc.* 2002, *124*, 2894–2902.

[11] Kwon, H.-J., Smith, W. C., Xiang, L., Shen, B., Cloning and heterologous expression of the macrotetrolide biosynthetic gene cluster revealed a novel polyketide synthase that lacks an acyl carrier protein, *J. Am. Chem. Soc*. 2001, *123*, 3385–3386.

[12] Walczak, R. J., Nelson, M. E., Priestley, N. D., Nonactin biosynthesis: Disruption of the polyketide synthase genes, *nonKJ*, in *Streptomyces griseus* leads to an overall 96% decrease in macrotetrolide production, yet a net increase in nonactin analogues which incorporate isobutyrate, *J. Am. Chem. Soc*. 2001, *123*, 10415–10416.

[13] Kwon, H.-J., Smith, W. C., Scharon, J., Hwang, S. H. *et al*., C-O bond formation in polyketide synthases, *Science* 2002, *297*, 1327–1330.

[14] Cox, J. E., Priestley, N. D., Nonactin biosynthesis: The product of the resistance gene degrades nonactin stereospecifically to form Homochiral nonactate dimers, *J. Am. Chem. Soc.* 2005, *127*, 7976–7977.

[15] Gerlach, H., Oertle, K., Thalmann, A., Servi, S., Synthese des Nonactins, *Helv. Chim. Acta* 1975, *58*, 2036–2043.

[16] Schmidt, U., Gombos, J., Haslinger, E., Zak, H., Hochstereoselektive Totalsynthese des Nonactins, *Chem. Ber*. 1976, *109*, 2628–2644.

[17] Gombos, J., Haslinger, E., Zak, H., Schmidt, U., Gezielte Synthese des Nonactins, *Tetrahedron Lett*., 1975, *16,* 3391–3394.

[18] Bartlett, P. A., Meadows, J. D., Ottow, E., Enantiodivergent syntheses of (+)- and (–)-nonactic acid and total synthesis of nonactin, *J. Am. Chem. Soc,* 1984, *106*, 5304–5311.

[19] Fleming, I., Ghosh, S. K., Stereocontrol in organic synthesis using silicon-containing compounds. A synthesis of nonactin, *J. Chem. Soc.Perkin I* 1998, 2733–2747.

[20] Bühlmann, P., Pretsch, E., Bakker, E., Carrier-based ion-selective electrodes and bulk optodes. 2. Ionophores for potentiometric and optical sensors, *Chem. Rev*. 1998, *98*, 1593–1687.

[21] Wang, G., Cole, R. B., Effect of solution ionic strength on analyte charge state distributions in positive and negative ion electrospray mass spectrometry, *Anal. Chem*. 1994, *66*, 3702–3708.

[22] Ho, Y. P., Huang, P. C., Deng, K. H., Metal ion complexes in the structural analysis of phospholipids by electrospray ionization tandem mass spectrometry, *Rapid Commun. Mass Spectrom*. 2003, *17*, 114–121.

[23] Antonopoulos, A., Bonnet, P., Botek, E., Debrun, J. L. *et al*., Study of the attachment of Na^+ on glucose and on some of its methylated derivatives, *Rapid Commun. Mass Spectrom*. 2003, *17*,122–125

[24] Emmert, J., Pfluger, M., Wahl, F., Influence of Na and K concentration in solvents on mass spectra of peptides in LC/ESI-MS, *LC/GC Europe* 2004, *17*, 646–649.

[25] Kaiser, P., Kramer, U., Meissner, D., Kress, M. *et al*., Determination of the cardiac glycosides digoxin and digitoxin by liquid chromatography combined with isotope-dilution mass spectrometry (LC-IDMS) – a candidate reference measurement procedure, *Clin. Lab*. 2003, *49*, 329–343.

[26] Schneider, R. P., Lynch, M. J., Ericson, J. F., Fouda, H. G., Electrospray ionization mass spectrometry of semduramicin and other polyether ionophores *Anal. Chem*. 1991, *63,* 1789–1794.

[27] Shen, J., Brodbelt, J. S., Characterization of ionophore–metal complexes by infrared multiphoton photodissociation and collision activated dissociation in a quadrupole ion trap mass spectrometer, *Analyst* 2000, *125*, 641–650.

[28] Weissmann, K. J., Mutasynthesis – uniting chemistry and genetics for drug discovery, *Trends Biotechnol*. 2007, *26*, 139–142.