Journal of Industrial Microbiology & Biotechnology (2001) 27, 144–148

www.nature.com/jim

Simocyclinones: diversity of metabolites is dependent on fermentation conditions[3]

J Schimana[1], M Walker[2], A Zeeck[2] and H-P Fiedler[1]

[1]*Mikrobiologisches Institut, Universität Tübingen, Tübingen 72076, Germany;* [2]*Institut für Organische Chemie, Universität Göttingen, Göttingen 37077, Germany*

Simocyclinones, a novel group of angucyclinone antibiotics, are produced by *Streptomyces antibioticus* Tü 6040. The compounds show antibacterial and antitumor properties. In submerged cultivation, the production of simocyclinones is strongly dependent on the carbon and nitrogen sources used in a chemically defined medium. Productivity of distinct components and diversity of simocyclinone compounds are influenced by the medium composition. Four series of simocyclinone compounds were detected by high-performance liquid chromatography (HPLC) diode array detector (DAD) and HPLC electrospray ionization (ESI) mass spectrometry (MS) analysis, isolated and the structures determined by nuclear magnetic resonance (NMR) techniques. Under optimized conditions, simocyclinone D8 was produced in an amount of 300 mg l^{-1} and simocyclinone C4 in a concentration up to 50 mg l^{-1}. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 144–148.

Keywords: angucyclinone antibiotics; *Streptomyces antibioticus*; chemical diversity; fermentation

Introduction

Simocyclinones are a novel group of secondary metabolites produced by *Streptomyces antibioticus* Tü 6040, showing antibacterial and antitumor properties. The main compounds, simocyclinones D4 and D8, were isolated from the mycelium and consist of a C-glycosylated angucyclinone ring attached to a tetraene side-chain and a coumarin ring which are shown in Figure 1. Simocyclinones may be designated as natural hybrid antibiotics, combining structural elements from various groups of antibiotics. The aglycone is similar to the angucyclinone antibiotic aquayamycin [4], the tetraene side-chain is similar to fumagillin, a fungal antibiotic [5], and the coumarin ring system is similar to novobiocin and chlorobiocin, respectively [2].

In shake flasks, simocyclinones D4 and D8 are produced after an incubation period of 6 days in amounts of about 20 and 40 mg l^{-1}, respectively, using a complex medium that consisted of mannitol and soybean meal. During the course of improving simocyclinone production by optimizing fermentation conditions, a chemically defined medium was developed that allowed exact determination of growth parameters and consumption of carbon and nitrogen sources, and production of the secondary metabolites at the same time. This paper describes the influence of various carbon and nitrogen sources on the yield of the main component simocyclinone D8, on one hand, and on the structural diversity of simocyclinone components, on the other hand, which can be increased impressively by modifying the medium. Results concerning taxonomy of the producing strain, isolation and structure elucidation of simocyclinones will be reported in forthcoming papers.

Correspondence: H-P Fiedler, Mikrobiologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, Tübingen D-72076, Germany

[3]Art No. 14 on "Biosynthetic Capacities of Actinomycetes." Art No. 13: see Ref. [3].

Received 21 September 1999; accepted 20 January 2000

Materials and methods

Media

The complex medium for cultivation of *S. antibioticus* Tü 6040 consisted of (per liter tap water): mannitol 20 g, soybean meal 20 g (pH 7.5 prior to sterilisation). A basal chemically defined medium consisted of (per liter deionized water): NaCl 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, 2 ml trace element concentrate (pH 7.3 prior to sterilisation), in addition to a carbon source and a nitrogen source as specified below. Trace element concentration (per liter deionized water): $FeSO_4 \cdot 7H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.1 g, $MnSO_4 \cdot H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g. As carbon sources (per liter): glycerol 25 g, mannitol 25 g, starch 25 g, and as nitrogen sources (per liter): L-glutamine 5.84 g/1.46 g, L-arginine 1 g.

Fermentation

S. antibioticus Tü 6040 was cultivated in 10-l stirred tank fermenters (Biostat E, B Braun, Melsungen, Germany) and in 20-l fermenters equipped with an intensor system (b20, Giovanola, Monthey, Switzerland). The fermenters were inoculated with 5 vol% of shaking cultures, grown for 48 h in 500 ml Erlenmeyer flasks on a rotary shaker at 120 rpm and 27°C in the same medium (100 ml). Fermentations were carried out at 27°C with an aeration rate of 0.5 v/v/m and an agitation of 200 rpm in stirred tank fermenters, and 1000 rpm in intensor systems.

High-performance liquid chromatography (HPLC) analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode array detector (DAD) and HP 3D-DOS ChemStation (Hewlett-Packard, Waldbronn, Germany).

The fermentation broth (10 ml) was extracted with the same volume of ethyl acetate. After centrifugation, the organic layer was concentrated *in vacuo* to dryness and dissolved in 1 ml

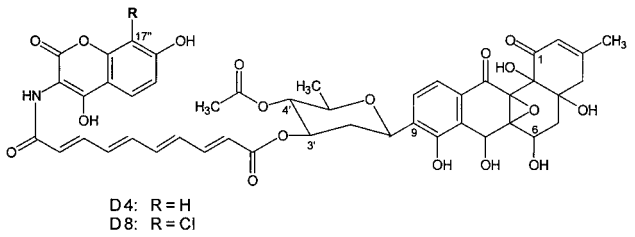

Figure 1 Structures of simocyclinones D4 and D8.

MeOH and 10 μl was injected on an HPLC column (4.6×125 mm^2, guard column 4.6×20 mm^2) filled with Nucleosil-100 C-18 (5 μm). Separation was done by a linear gradient using 0.1% phosphoric acid as solvent A and acetonitrile as solvent B and a gradient from 0% to 100% solvent B within 15 min at a flow rate of 2 ml min^{-1}.

Results

Optimizing simocyclinone D8 production

In cultivations in the complex medium used for screening purposes that consisted of mannitol and soybean meal, the main compound, simocyclinone D8, was produced at a maximum concentration of about 30 mg l^{-1} in 10-l fermenters. Using a basal chemically defined medium, a large variety of carbon sources was tested in combinations of amino acids and ammonium salts as nitrogen source for growth of *S. antibioticus* Tü 6040 and production of simocyclinone D8.

Glycerol (25 g l^{-1}) and L-glutamine (5.84 g l^{-1}) as carbon and nitrogen sources, respectively, were favourable for production mainly of simocyclinone D8 at a concentration of 80 mg l^{-1} which accumulated in the mycelium. Whereas a combination of starch (25 g l^{-1}) and L-glutamine (1.46 g l^{-1}) resulted in an increase in simocyclinone D8 production and led to a yield of 300 mg l^{-1} after a fermentation time of about 150 h (Figure 2).

Increasing diversity of simocyclinone compounds

Four types of simocyclinone compounds were found in the fermentation broth dependent on the carbon and nitrogen sources. The novel simocyclinone compounds were detected by their UV–visible spectra in HPLC-DAD analysis and were divided into series A–D, from which series D represents the original simocyclinones D4 and D8 produced. The four UV–visible spectra are shown in Figure 3.

The D-series was produced exclusively using glycerol (25 g l^{-1}) as carbon source and L-glutamine (5.84 g l^{-1}) as nitrogen source. Using a combination of mannitol (25 g l^{-1}) and L-arginine (1 g l^{-1}), besides a new member of the D-series, simocyclinone D7, simocyclinone A1 and two members of the B-series, simocyclinones B1 and B2, were detected in the culture filtrate. Using starch (25 g l^{-1}) as carbon source and L-glutamine (1.46 g l^{-1}) as nitrogen source, a further new series of simocyclinone compounds, C2 and C4, was produced of which C4 was a main component in the fermentation broth, reaching an amount of 50 mg l^{-1}, whereas simocyclinone D8 was produced at a concentration of 300 mg l^{-1}. The variation in the metabolite pattern depending on carbon and nitrogen sources of the medium is shown in Figure 4.

Structures and biogenesis of simocyclinone compounds

New members of the group of simocyclinones were detected by HPLC-DAD and HPLC electrospray ionization (ESI) mass spectrometry (MS) analysis in extracts of the fermentation broth. They were isolated by chromatographic methods and the structures elucidated by nuclear magnetic resonance (NMR) techniques.

The A-series of simocyclinones consists of the polyketide-derived aglycone, having a hydroxyl modification in position 6 of ring B in the case of simocyclinone A2. The B-series represents the glycosylated A-series of simocyclinones, showing an additional variation in the sugar moiety by an acetylation. In the C-series, the molecules are completed by a tetraene side-chain, which is also derived from polyketide biosynthesis. Finally, the D-series contains a coumarin ring system attached by an amide bond, and is derived biosynthetically from tyrosine as in novobiocin and

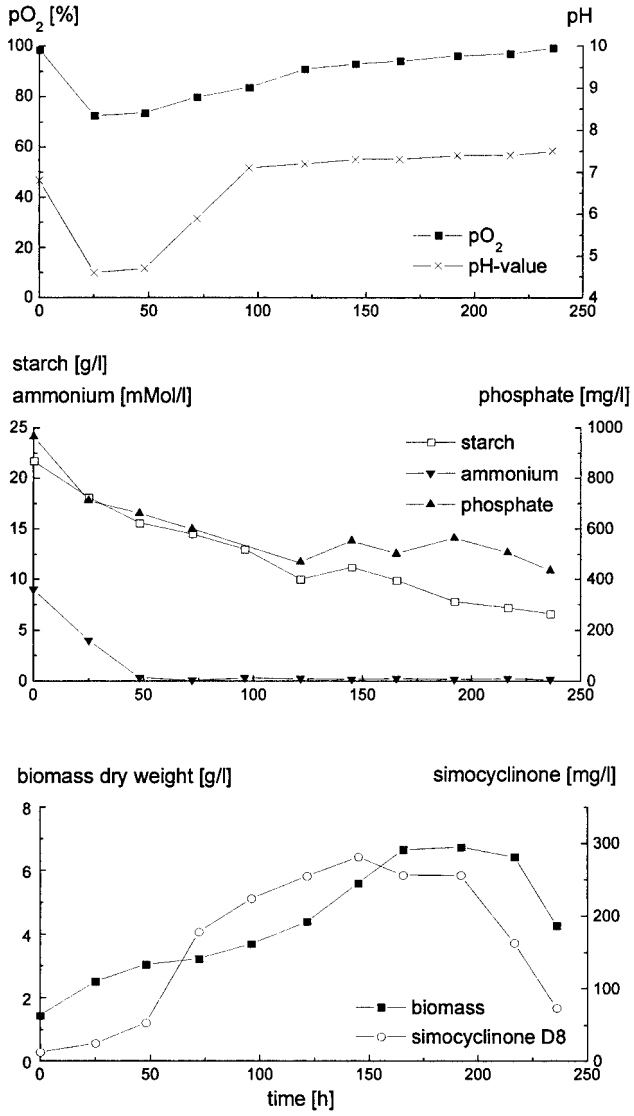

Figure 2 Ten-liter fermentation of *S. antibioticus* Tü 6040 in a chemically defined medium containing starch and L-glutamine as carbon and nitrogen sources, respectively.

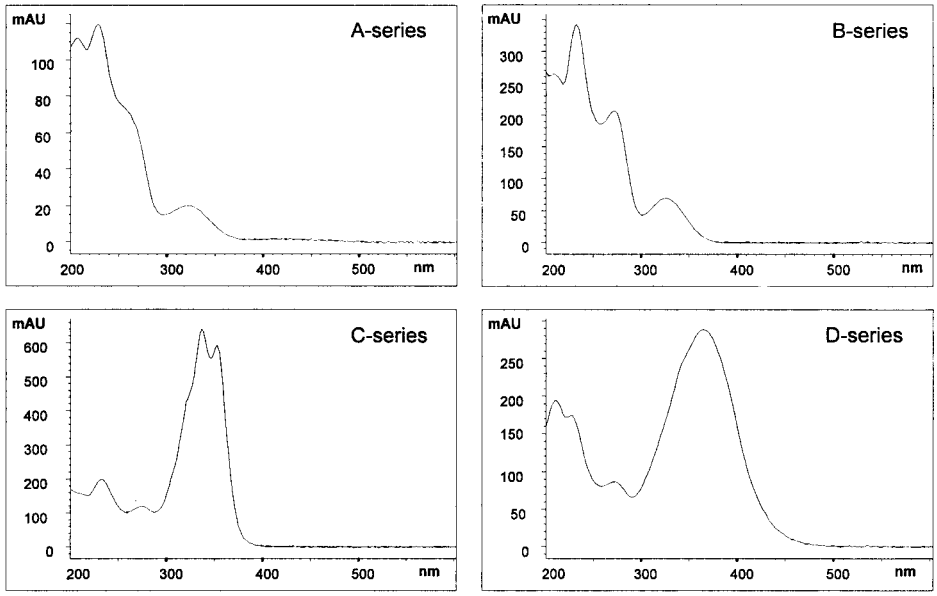

Figure 3 UV–visible spectra of simocyclinones of the A-, B-, C- and D-series, monitored during HPLC-DAD analysis.

chlorobiocin. The structural variety of simocyclinones is summarized in Figure 5.

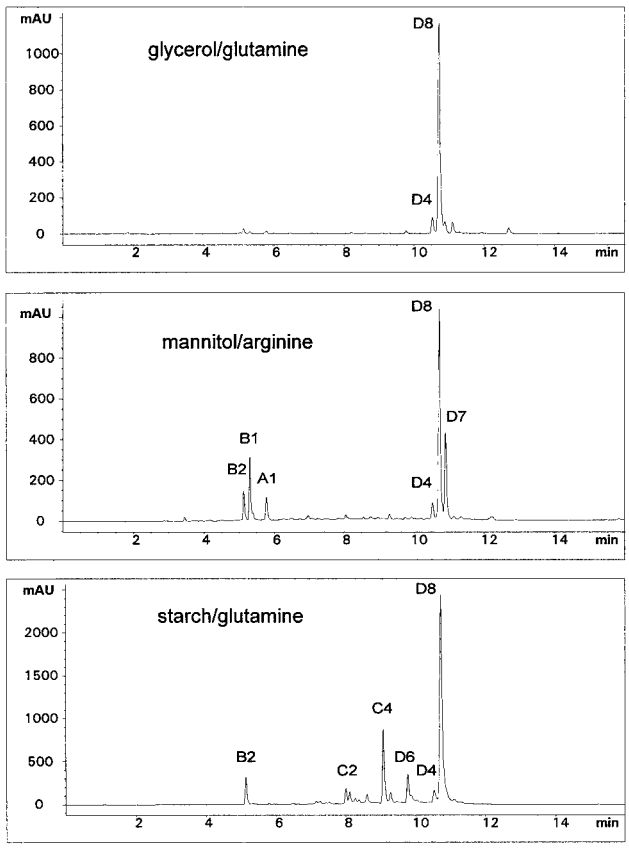

Figure 4 Diversity of simocyclinones produced in a chemically defined medium depending on the carbon and nitrogen sources. HPLC analysis of extracts from the culture broth of *S. antibioticus* Tü 6040, monitored at 230 nm. Fermentation scale, 10 l.

Discussion

Modifications of the physiological conditions for a strain, such as variation of the medium composition and fermentation conditions, are still efficient tools for increasing productivity for a distinct component and increasing the natural diversity of secondary metabolites. In the case of *S. antibioticus* Tü 6040, production can be turned towards one of the four different simocyclinone series dependent on the carbon and nitrogen source which are added to a simple chemically defined medium. The ratio of compound D8 to D4, which is the dechlorinated D8 molecule, is normally shifted strongly in the direction of D8 in all media that contain NaCl. However, when NaCl is replaced by NaI, the ratio of the two compounds is shifted completely towards the dechlorinated molecule simocyclinone D4 because iodine cannot be added to the coumarin ring.

New members of the simocyclinone family were found by using altered carbon and nitrogen sources, resulting in three further series of simocyclinone compounds. When starch was used as carbon source and L-glutamine as nitrogen source, the C-series was detected in the culture filtrate with simocyclinone C4 as a main component, reaching a maximum of 50 mg l^{-1} in the culture broth.

All compounds were detected in mycelium extracts, culture filtrate extracts or in extracts of the whole culture broth by HPLC-DAD analysis and were characterized regarding their UV–visible spectra [1]. This technique permitted the classification of simocyclinones into four series. In addition, HPLC-ESI-MS analysis revealed the molecular mass of each compound and enabled, together with the UV spectral data, a reliable prognosis of the structures during the fermentation process, which was then confirmed by isolation of the compounds and by NMR data. By these techniques, a nearly complete survey on the biogenesis of the various simocyclinones was obtained, which is shown in Figure 6. A parallel stream of compounds is derived from simocyclinones A1 and A2, which differ in the hydroxylated position 6 of the angucyclinone ring in case of A2. Further parallel streams of compounds are derived by acetylation of the sugar, olivose, or by

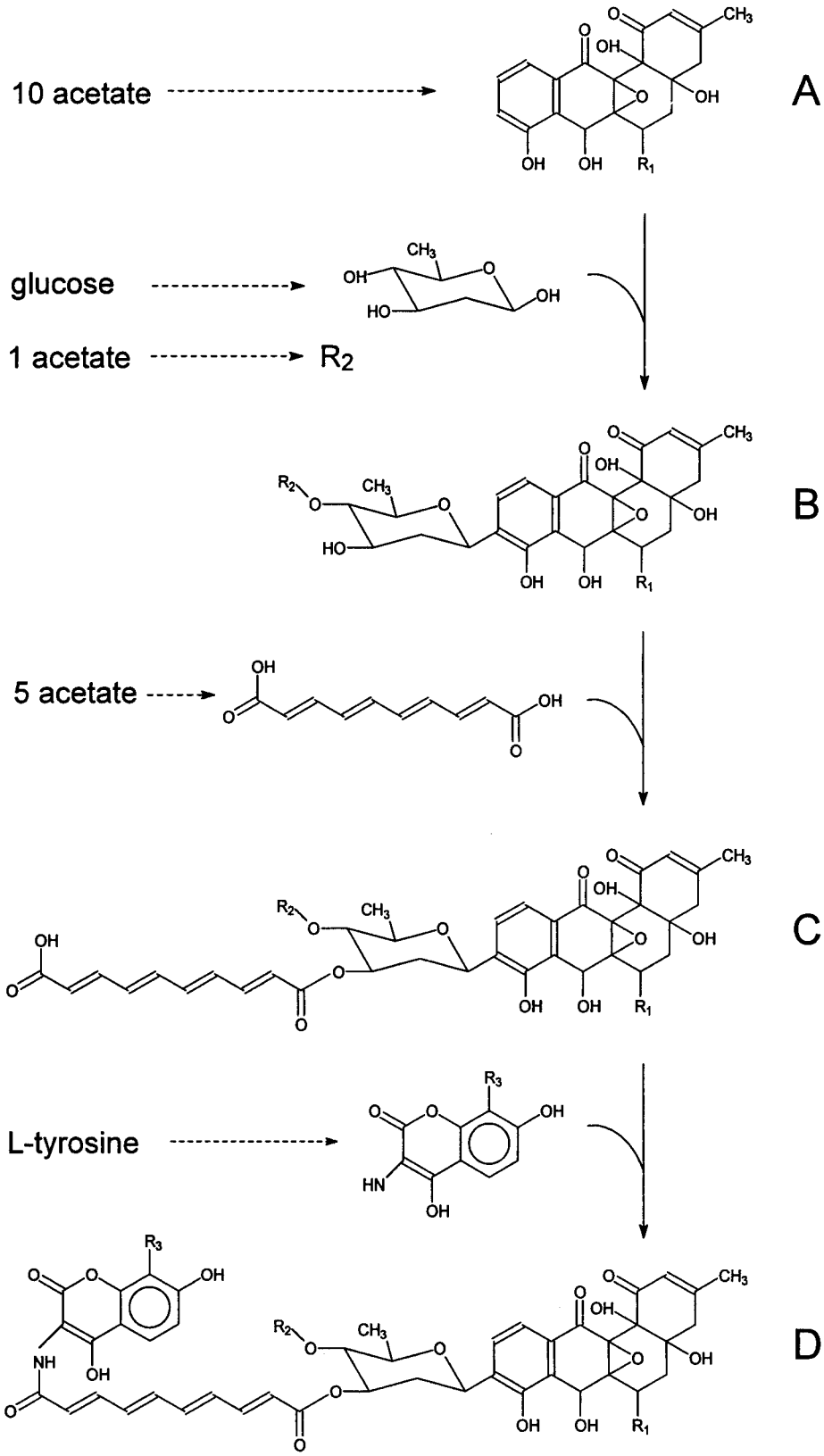

Figure 5 Diversity of simocyclinone structures created by *S. antibioticus* Tü 6040 as intermediates during biosynthesis of the simocyclinone D-series.

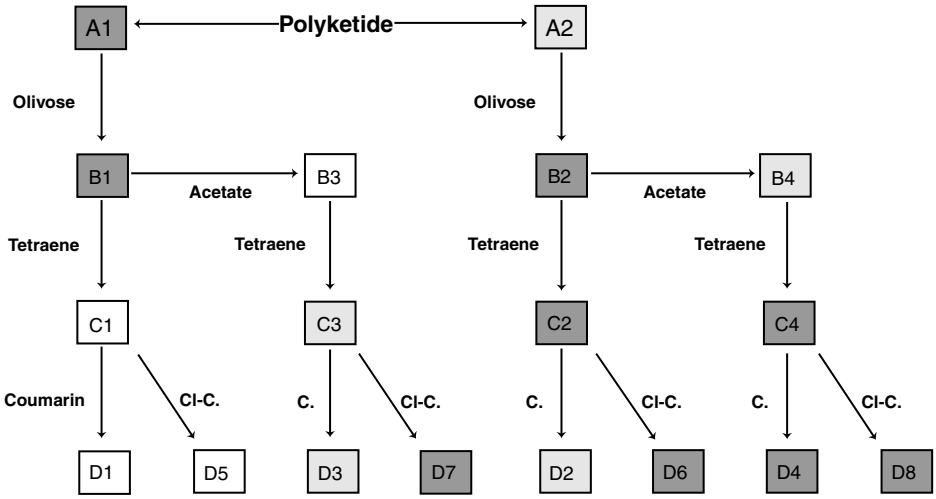

Figure 6 Hypothetical biogenesis scheme of simocyclinones from the A-, B-, C- and D-series. The structures of the dark grey compounds were determined by NMR, the light grey compounds were characterized by MS, the white compounds are hypothetical. C = coumarin, Cl-C = chlorocoumarin.

chlorination of the coumarin ring, yielding a total of 18 simocyclinone compounds from which nine compound structures were determined by NMR and five further compounds were characterized by MS analysis. Four of the simocyclinones shown in Figure 6 are so far hypothetical.

Acknowledgements

J.S. thanks Graduiertenkolleg Mikrobiologie, Universität Tübingen, for a doctoral scholarship. The authors thank R Süßmuth, Institut für Organische Chemie, Universität Tübingen, for HPLC-ESI-MS analyses, and BMBF Bundes-republik Deutschland and BASF AG, Ludwigshafen (FKZ 0310722) for financial support.

References

1 Fiedler H-P. 1993. Biosynthetic capacities of actinomycetes: 1. Screening for secondary metabolites by HPLC and UV–visible absorbance spectral libraries. *Nat Prod Lett* 2: 119–128.
2 Hinman JW, EL Caron and H Hoeksema. 1957. The structure of novobiocin. *J Am Chem Soc* 79: 3789–3800.
3 Maier A, J Müller, P Schneider, H-P Fiedler, I Groth, FSK Tayman, F Teltschik, C Günther and G Bringmann. 1999. (2*E*,4*Z*)-Decadienoic acid and (2*E*,4*Z*,7*Z*)-decatrienoic acid, two herbicidal metabolites from *Streptomyces viridochromogenes* Tü 6105. *Pesticide Sci* 53: 733–739.
4 Sezaki M, T Hara, S Ayukawa, T Takeuchi, Y Okami, M Hamada, T Nagatsu and H Umezawa. 1968. Studies on a new antibiotic pigment, aquayamycin. *J Antibiot* 21: 91–97.
5 Tarbell DS, RM Carman, DD Chapman, KR Huffman and NJ McCorkindale. 1960. The structure of fumagillin. *J Am Chem Soc* 82: 1005–1006.