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Liquid chromatographic method for separation and determination of elaiophylin in biotechnology processes of salinomycin production*

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Abstract: An LC method for the quick and precise quantitative determination of elaiophylin in biotechnology processes of salinomycin production was prepared. A mobile phase of methanol 0.04 M diammonium hydrogen phosphate pH 6.0–ethyl acetate (55:35:10, v/v/v) and an elevated column temperature optimized the chromatography. Separation of elaiophylin was obtained in 4–6 min. Positive identification and peak purity of elaiophylin have been carried out by photodiode array (PDA) detection. An agreement between the PDA spectra of samples and the elaiophylin standard were obtained.

Keywords: *Elaiophylin; LC method development; photodiode array (PDA) detection; salinomycin production process.*

Introduction

The macrodiolide antibiotic elaiophylin also known as azalomycin B or salbomycin was originally isolated from cultures of *Streptomyces melanosporus* and exhibits anthelmintic activity as well as activity against gram-positive bacteria and protozoa. Later, elaiophylin was also isolated from other strains of *Streptomyces*, such as *Streptomyces hygroscopicus* var. *azalomyceticus* [1–3] and *Streptomyces violaceoniger* [4]. After its isolation [1], the main physical and chemical properties of elaiophylin were determined and elaiophylin was declared a member of the unusual group of symmetrical, 16-membered macrodiolides [5].

Recently, the appearance of elaiophylin as an unwanted co-metabolite was detected in some industrial fermentations of the polyether antibiotic salinomycin, produced by *Streptomyces albus*. Elimination of the elaiophylin from the salinomycin production process was a goal of a particular *Streptomyces albus* production strain improvement, and a pure salinomycin strain was isolated after random screening on *Streptomyces albus* ATCC 21838. As abundant and complex byproducts are usually present in the unrefined antibiotics after finished biotechnology processes of the main component production, chromatographic separation methods are necessary for the determination of individual components. However, previously published thin-layer bioautographic [2] and paper bioautographic [1] methods are unsuitable for the precise quantitative elaiophylin determination. Thus, a simple, rapid and reliable LC method utilizing photodiode array (PDA) detection has been developed and is described in this paper.

Experimental

Chemicals and samples

Methanol, ethyl acetate and diammonium hydrogen phosphate were of analytical reagent grade (Kemika, Zagreb, Croatia). Water was deionized and distilled. For the quantitative determinations, elaiophylin had to be isolated and purified in an amount sufficient for the preparation of a working standard. For this purpose, salinomycin feed grade batch previously determined to contain elaiophylin were used. From this material, elaiophylin was selectively extracted with ethyl acetate and hexane and isolated in crystalline form. Confirmation LC tests utilizing PDA detection and quantitative determination compared to the reference elaiophylin standard (LUFA

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Augustenberg and LUFA Kiel, Germany) was done on this purified elaiophyliln material. The results corresponded to the reference elaiophylin material and consequently this substance was used as the working standard material. Different process samples of salinomycin (fermenation broths, feed grade preparations) were supplied by Krka Pharmaceuticals (Novo mesto, Slovenia). Some other commercial samples of 6–12% (w/w) salinomycin feed grade (Kaken Chemical Co. Ltd, Tokyo, Japan; Hoechst, Frankfurt am Main, Germany; Pharmachim, Sofia, Bulgaria) were obtained.

Instrumentation and operating conditions

The LC instrumentation for the elaiophylin determination consisted of an LKB pump 2150, an LKB variable wavelength monitor 2151, operated at 252 nm and an LKB column oven 2155. In the method development as well as in peak purity tests and UV spectra comparisons, an LKB 2140 rapid spectral detector (PDA) (LKB, Bromma, Sweden) was used. Separations were made on a Chromspher C18 column (5 μm, 150 × 4.6 mm i.d.) and reversed-phase pelicular precolumn (75 × 4.6 mm i.d.) coupled with the PDA detector. Routine analysis with optimized LC conditions were made on a ChromSep Chromspher C18 cartridge (5 μm, 100 × 3 mm i.d.) coupled with a Chromguard reversed-phase column (10 × 3 mm i.d.) (Chrompack, Middleburg, Netherlands). Flow rates were 1.0 and 0.5 ml min^{-1}, respectively, and the temperature of separation was 35°C. Samples were injected through a Rheodyne Model 7125 injector fitted with 20 μl fixed loop (Rheodyne, Inc., Cotati, CA, USA). Integration was based on peak area or peak height measurement on an LKB 2220 recording integrator. An external standard procedure was used for quantitative determinations of elaiophylin.

Sample preparation

The stock solution of elaiophylin was prepared by dissolving an accurately weighed elaiophylin standard in methanol and further diluting in mobile phase to the concentration of 1000 μg ml^{-1}. This solution was stable for 30 days when refrigerated at 4°C. From different samples, elaiophylin was extracted with methanol in an ultrasonic bath for 2 min and then on the rotary shaker for an additional 30 min. The supernatant liquid was further diluted to an appropriate concentration with mobile phase. Dilutions of 3–5 μg ml^{-1} elaiophylin were usually chromatographed after filtration through a 0.45 μm membrane.

Results and Discussion

Optimization of LC parameters

Due to the absence of an elaiophylin standard at the time of LC method development, identification of elaiophylin was carried out with PDA detection at 220–300 nm where the elaiophylin absorption [2] was expected. The UV absorbing components of different salinomycin feed grade preparations were tested on the RP ODS column without post-column derivatization reaction devices (salinomycin remained undetectable) under the same conditions as described in ref. 6. In this case the mobile phase was mainly methanol (methanol–water; 94:6, v/v). Under these conditions, it was found that in some of tested samples one component (Fig. 1a) exhibited a UV spectra (Fig. 1b) that was comparable to the literature data for the elaiophylin UV spectra in methanol with maximum absorption at 254–256 nm [2]. It was a first indication that elaiophylin is present in some salinomycin products. But resolution of its peak from other components was poor and there was a high background due to the residual matrix.

Better separation of elaiophylin from other unidentified components was obtained by increasing the water content of the mobile phase. A mobile phase consisting of methanol–water (78:22, v/v) gave satisfactory separation (Fig. 2a and b). Aqueous solutions of elaiophylin showed the best stability at pH 6.0, thus diammonium hydrogen phosphate buffer (0.025 M, pH 6.0) was added to the mobile phase. Additionally, phosphate buffer increased the efficiency of elaiophylin separation and consequently the separation factor (α) and resolution (*Rs*) were increased (Fig. 2b). An increase of the aqueous buffer fraction in the mobile phase over 22% (v/v) greatly increased the retention time of elaiophylin but the addition of ethyl acetate helped to reduce the elaiophyllin elution time. When up to 10% (v/v) ethyl acetate was added, further increase of the aqueous fraction to 35% (v/v) was possible with appropriate elaiophylin retention. Because the increased aqueous fraction permitted a higher concentration of phosphate buffer up to 0.04 M, an additional improve-

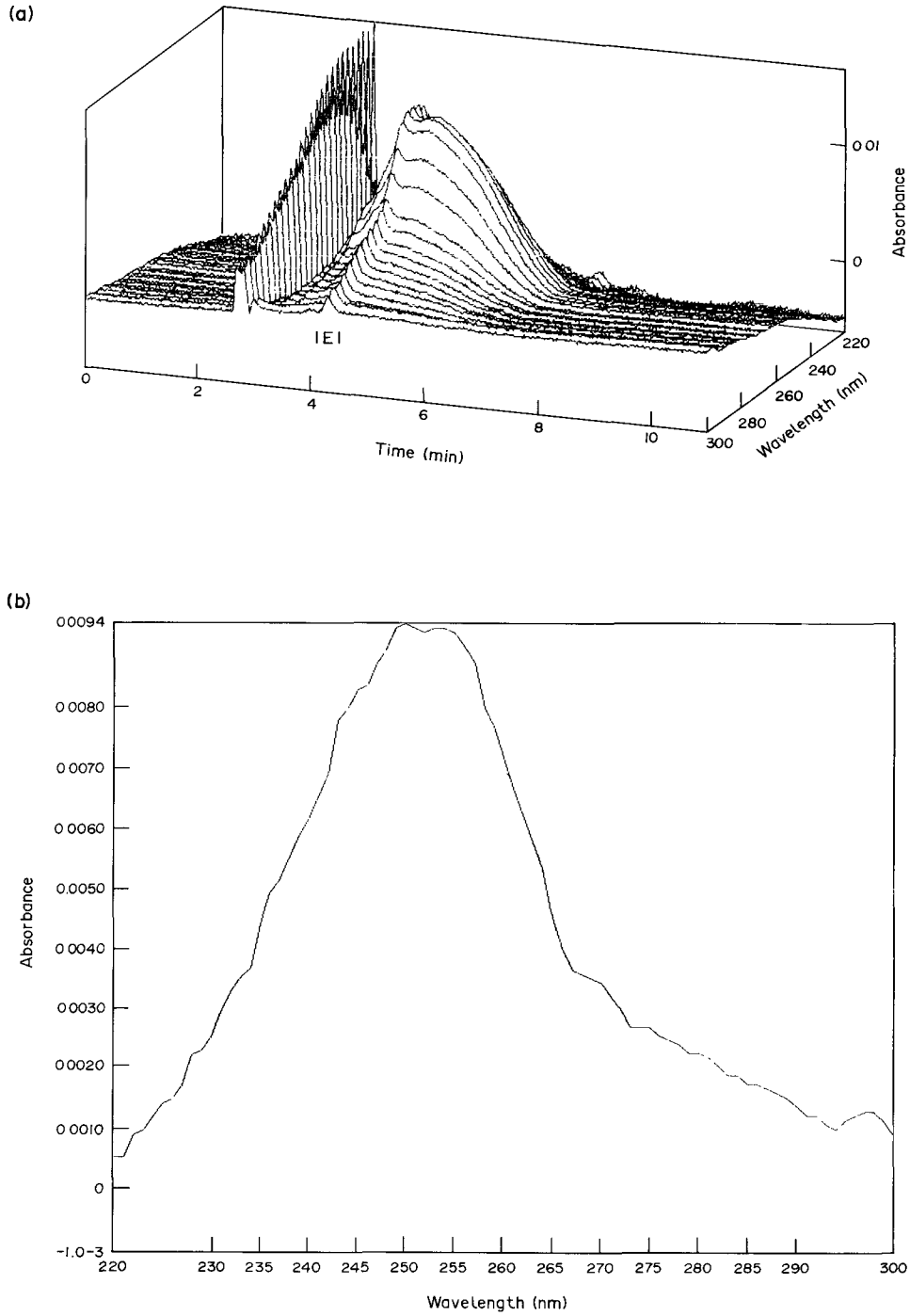

Figure 1
(a) Diode array topogram of the salinomycin 12% (w/w) feed grade preparation suspected to contain elaiophylin (E). Mobile phase: methanol–water (94:6, v/v). The water contained 1.5% (v/v) glacial acetic acid. For other conditions see text. (b) UV spectra at the apex position of component (E).

ment of resolution and sensitivity for the elaiophylin peak was obtained (Fig. 2c). Buffer concentration higher than 0.04 M can cause salt precipitation and blocking of the LC system. When all components in the mobile phase constitution and factors influencing elaiophylin separation were optimized, the mobile phase used was: methanol–0.04 M diammonium hydrogen phosphate (pH 6.0)–ethyl acetate (55:35:10, v/v/v). The UV spectra of elaiophylin in this mobile phase were significantly different than in methanol with

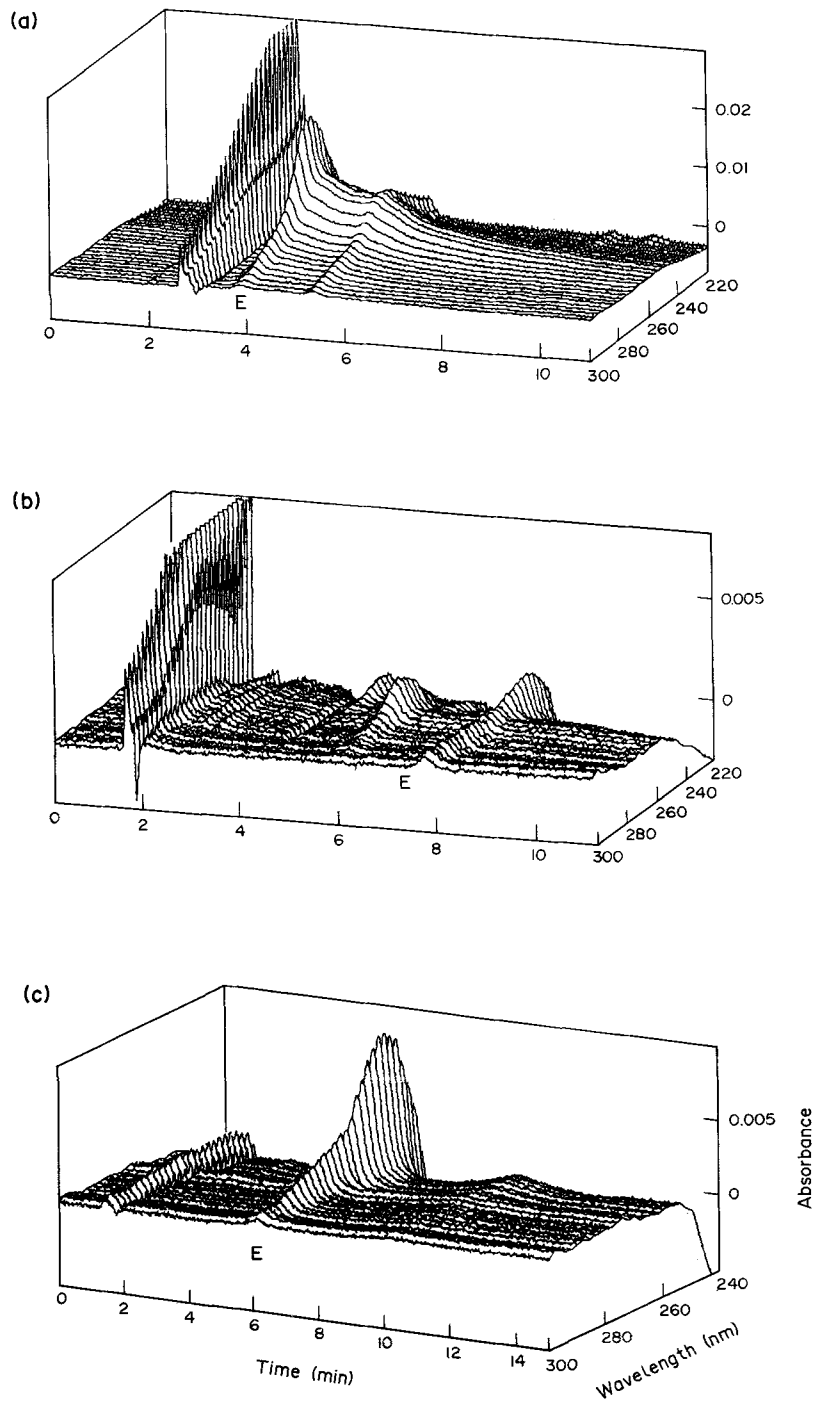

Figure 2
Improvement of the elaiophylin (E) LC separation by modifying the mobile phase preparation. (a) Mobile phase: methanol–water (90:10, v/v). (b) Mobile phase: methanol–0.025 M $(NH_4)_2HPO_4$, pH 6.0 (78:22, v/v). (c) Mobile phase: methanol–0.04 M $(NH_4)_2HPO_4$, pH 6.0–ethylacetate (55:35:10, v/v/v).

absorbance at 240–300 nm while the absorption maximum remained unchanged at 254–256 nm (Figs 2c and 3b). Influence of temperature on the elaiophylin separation was also tested and it was found that elevated temperatures (35–45°C) enhanced the efficiency of the separation (capacity factor k' decreased). Appearance of elaiophylin in all of the experiments during the LC method improvement was followed by the comparison of samples with standard elaiophylin preparation. As the retention time of the analyte was the

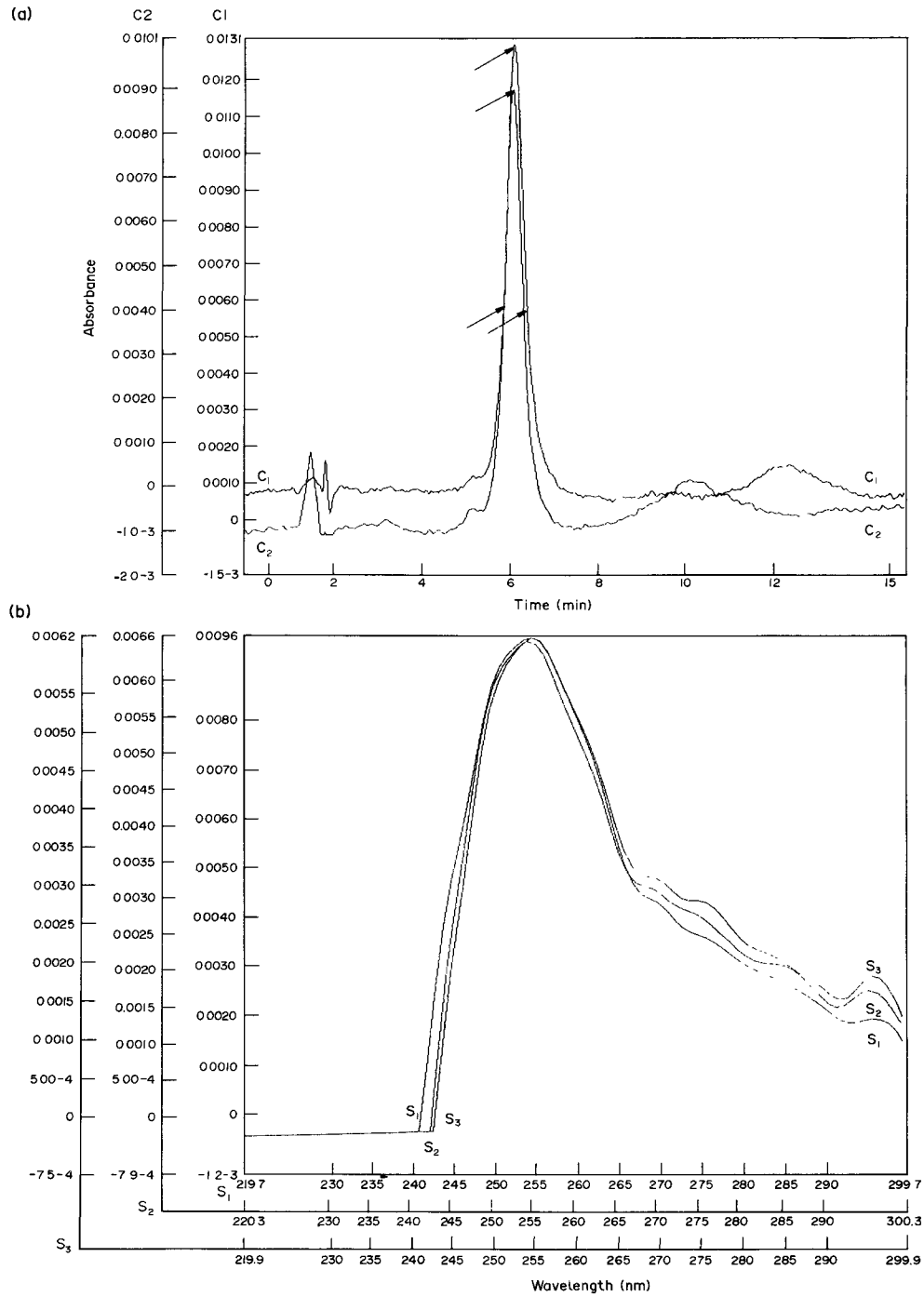

Figure 3
(a) Chromatograms obtained by the optimized LC condition. (C1) = elaiophylin standard; (C2) = real sample of elaiophylin positive salinomycin feed grade. Detection: 252 nm. Points of peak purity tests are indicated. (b) Peak purity test of elaiophylin peak from real sample of salinomycin feed grade. Spectral curves are from: upslope (S1), apex (S2) and downslope (S3) of the peak. Correlation factors for curves: S1–S2 = 0.99696, S2–S3 = 0.99297 and S1–S3 = 0.99595.

primary means of identification, the column oven was also helpful in stabilizing retention time fluctuation.

With optimized LC conditions, the minimum detection quantity of elaiophylin was determined at low attenuation of signal height to noise ratio = 3. The limit of detection was 0.05 μg ml^{-1}. Linear response was obtained over the concentration range of 0.2–30 μg ml^{-1}. Consequently, the limit of quantitation

expressed as the smallest elaiophylin peak whose area can be measured with acceptable precision, was 0.2 μg ml^{-1}. Repeated elaiophylin analysis in the same sample of salinomycin feed grade gave an RSD of 2.93% ($n = 6$).

The peak purity of the elaiophylin in the optimized LC conditions was checked by PDA detection. Comparisons of the absorbance spectra extracted from the stored data at the upslope, apex and downslope of the peak was tested. Correlation factors of these UV spectra curves were between 0.993 and 0.997 and this fact indicated that the elaiophylin peak of the samples was pure (Fig. 3). Comparison of the elaiophylin UV spectra to the spectra of the elaiophylin standard has also been carried out. The selected and normalized PDA spectra of the samples were not significantly different from the corresponding spectra of elaiophylin standard.

Analysis of samples

When commercially available samples of salinomycin feed grade materials from different manufacturers were tested for elaiophylin by the LC method, some of them appeared to be elaiophylin positive. Elaiophylin production of *Steptomyces albus* as the salinomycin-producing strain needed to be investigated. For this purpose, different salinomycin-producing strains of *Streptomyces albus* were tested. It was realized that some of them, as well as *Streptomyces albus* ATCC 21838 mentioned in the salinomycin patents [7, 8], exhibited elaiophylin-producing capabilities along with its salinomycin production. Since most salinomycin-producing strains were previously derived from *Streptomyces albus* ATCC 21838, elimination of elaiophylin as an unwanted co-metabolite was required due to its undesirable properties and difficulties in removal during downstream processing of salinomycin production. In further work, following several rounds of artificially- or naturally-produced mutant strains of *Streptomyces albus*, a pure salinomycin-producing strain as well as a pure elaiophylin-producing strain were selected.

Oil is essential as the main carbon source in the nutrition media for a high yield of salinomycin biosynthesis [9, 10]. But during this work it was found that oil was equally important for the high elaiophylin yield. When pure producing strains were isolated, significantly higher yields of salinomycin and elaiophylin were obtained due to the complete oil consumption in the single biosynthetic pathway.

References

[1] M. Arai, *J. Antibiot., Ser. A* **8**, 46–50 (1959).
[2] V. Ivanova, R. Rachev, R. Gesheva, M. Darakcieva, P. Panayotov, D. Belomejova, T. Ivanova and R. Genkova, *Acta Microbiol. Bulg.* **12**, 24–31 (1983).
[3] S. Takahashi, M. Arai and E. Ohki, *Chem. Pharm. Bull.* **15**, 1651–1656 (1967).
[4] H.P. Fiedler, W. Worner, H. Zahner, H.P. Kaiser, W. Keller-Schierlein and A. Muller, *J. Antibiot.* **34**, 1107–1118 (1981).
[5] H.P. Kaiser and W. Keller-Schierlein, *Helv. Chim. Acta* **64**, 407–424 (1981).
[6] M. Sokolić and M. Pokorny, *J. Pharm. Biomed. Anal.* **9**, 1047–1053 (1991).
[7] United States Patent 3,857,948 dec. 31 (1974).
[8] German patent application DE 32 482 80 A1 (1984).
[9] Y. Miyazaki, M. Shibuya, H. Sugawara, O. Kawaguchi, C. Hirose, J. Nagatsu and S. Esumi, *J. Antibiot.* **27**, 814–821 (1974).
[10] N. Otake, in *Biotechnology of Industrial Antibiotics* (E.J. Vandame, Ed.), pp. 721–742. Marcel Dekker, New York (1983).

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