Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 68–72

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

LC–NMR and LC–MS identification of an impurity in a novel antifungal drug icofungipen

Predrag Novak[a,*], Predrag Tepeš[b], Marina Ilijaš[c], Ines Fistrić[c], Igor Bratoš[d], Amir Avdagić[d], Zdenko Hameršak[e], Vesna Gabelica Marković[c], Miljenko Dumić[d]

[a] Department of Chemistry, Faculty of Natural Sciences, University of Zagreb, Horvatovac 102a, HR-10000 Zagreb, Croatia
[b] Faculty of Geotechnical Engineering, Hallerova aleja 7, HR-42000 Varaždin, Croatia
[c] GlaxoSmithKline Research Center Zagreb Ltd., Prilaz baruna Filipovića 29, HR-10000 Zagreb, Croatia
[d] PLIVA- R&D Croatia Ltd, Prilaz baruna Filipovića 29, HR-10000 Zagreb, Croatia
[e] Ruđer Bošković Institute, Bijenička 57, HR-10000 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 5 February 2009
Received in revised form 12 March 2009
Accepted 13 March 2009
Available online 25 March 2009

Keywords:
Icofungipen
Impurity
Liquid chromatography–nuclear magnetic resonance spectroscopy
Liquid chromatography–mass spectrometry

ABSTRACT

Successful use of LC–NMR and LC–MS for rapid identification of an impurity in a novel antifungal drug icofungipen has been demonstrated. Complementary information obtained from the two methods made it possible to determine the structure of **A1** prior to its isolation and purification. Stop-flow LC–NMR (^{1}H and DQFCOSY), LC–MS and LC–MS/MS spectra have shown that **A1** is structurally related to icofungipen. It was later isolated and prepared synthetically and its structure was corroborated by high-resolution NMR spectroscopy.

1. Introduction

Icofungipen (formerly PLD-118, BAY 10-8888) is a novel, orally bioavailable antifungal drug, under development for the treatment of *Candida* infections. Owing to the limitations of the existing antifungal drugs there is a continuing need for new classes of antifungal compounds with increased efficacy and tolerability. Icofungipen, **1** (Scheme 1) is a synthetic derivative of the naturally occurring β-amino acid cispentacin possessing a novel mechanism of action. Icofungipen was shown to inhibit isoleucyl-tRNA synthetase resulting in the inhibition of protein biosynthesis and of fungal cell growth [1–6]. Owing to its specific activity against *Candida* spp. including azole-resistant strains *in vitro* and *in vivo* and also favourable pharmacokinetics, solubility and safety it was selected for clinical trials.

We have recently reported a strategy to combine LC–MS and LC–NMR methods as an effective tool for characterization of impurities and degradation products in drug molecules [7–9]. LC–NMR and LC–MS methods are now widely accepted and used to analyze compound mixtures and metabolites. In this paper we report on the application of LC–NMR and LC–MS methods for rapid identification and characterization of an unknown impurity **A1** in icofungipen. Fast and reliable identification and structure elucidation of impurities present in final drug substances or drug candidates in higher developmental phases represent a primary task for analytical chemists working in pharmaceutical industry, bearing in mind strong regulations imposed by regulatory bodies. The impurity **A1** was formed during the preparation of icofungipen and has not yet been reported in the literature.

2. Experimental

2.1. Materials

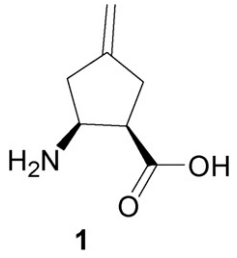
Acetonitrile for chromatography (gradient grade, MERCK, Darmstadt, Germany), deuterium oxide (min. 99.9% pure, Cambridge Isotope Laboratories, Andover, USA) and water Milli Q purity (Millipore filters, Billerica, MA, USA) were used.

2.2. Sample preparation

Water solutions (2 mg/ml) of the samples were prepared for the LC and LC–MS analyses. An aliquot of the water solution was diluted by acetonitrile–water mixture (50:50, v/v), to the final concentra-

* Corresponding author. Tel.: +385 1 4819283; fax: +385 1 372 1570.
E-mail address: pnovak@chem.pmf.hr (P. Novak).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.03.017



1

Scheme 1.

tion of 0.2 mg/ml, and then used for LC–MS/MS analysis. LC–NMR was performed with 12–120 mg/ml D_2O solution of the samples for ^1H and COSY LC–NMR experiments.

2.3. Liqiud chromatography

Chromatography was optimized on an ET 250/3 Nucleosil 100-5 C18 AB chromatography column using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) comprising a binary pump, autosampler, column compartment and diode array detector (DAD) set at 210 and 254 nm. HPLC analyses were performed in both isocratic and gradient modes. The best conditions were reached at isocratic mode with mobile phase composition of 25% acetonitrile and 75% H_2O at the flow rate of 0.5 ml/min over 20 min. Those conditions were applied further in LC–MS and LC–NMR experiments.

2.4. LC–MS and LC–MS/MS measurements

The LC–MS system comprised an Agilent 1100 HPLC system (Agilent Technologies Waldbronn, Germany) consisting of a quaternary pump, autosampler, column compartment and a DAD detector and a Micromass Platform LCZ (Waters, Milford, USA) single quadrupole mass spectrometer, operating in Electrospray ionization (ESI) positive ion mode. The HPLC parameters were the same as described in the previous section. The MS parameters were as follows: capillary voltage 3.0 kV, cone voltage 20 V, desolvation temperature 250 °C, source temperature 100 °C, nitrogen flow 420 l/h.

The LC–MS/MS measurements were carried out on a system comprising a Surveyor HPLC system and a LCQ Deca ion trap mass spectrometer (both ThermoFinnigan San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The HPLC parameters were as already described and MS parameters were as follows: scan 50–400 amu (for full scan), precursor ion $m/z = 258$ (for MS/MS function), spray voltage 3.0 kV, sheat gas 80 units, aux. gas 10 units, capillary voltage 20 V and capillary temperature 250 °C.

2.5. LC–NMR measurements

The stop-flow LC–NMR spectra were acquired on an Avance DRX500 spectrometer (BrukerBiospin, Rheinstetten, Germany) coupled to a Bruker LC 22 pump, with Bischoff Lambda 1010 UV-detector operating at 220 nm and a BSFU Bruker Stop Flow Unit. The same column as that described in Section 2.3 was used.

An isocratic composition of 75% D_2O and 25% acetonitrile was used. The flow rate was 0.5 ml/min. We used different injection volumes (0.1–1 ml) for ^1H and COSY LC–NMR experiments. In order to obtain reproducible and reliable COSY LC–NMR spectra (**A1** was present at very low amounts) we tried to achieve as high concentration as possible and not to spoil the chromatographic separation. An inverse 4 mm detection $^1H/^{13}C$ flow probe (cell volume 120 μl) with z-gradient accessory was used.

One-dimensional NMR spectra were recorded using the WATERGATE and WET pulse sequences for suppression of the acetonitrile and HOD signals. Spectra were acquired with a 6000 Hz spectral window and 64K data points, which gave the digital resolution of 0.4 Hz per point. 100–260 scans were accumulated to obtain appropriate signal-to-noise ratio.

Two-dimensional DQCOSY LC–NMR spectra were acquired with the spectral width of 6000 Hz in both dimensions and spectra were transformed into 2K data points with 512 increments and 64 scans. Data were processed using the unshifted sine square window function. Digital resolution was 1.5 Hz in both dimensions.

2.6. NMR measurements

One- and two-dimensional (^1H, APT, gCOSY, gHSQC and gHMBC) NMR spectra were recorded on the Avance DRX500 spectrometer using a 5 mm diameter inverse detection probe with z-gradient. The spectra were recorded in D_2O with the sample concentration of 25 mg/ml and TSPD as the internal standard. The standard spectral conditions for one-dimensional (^1H and APT) and two-dimensional (gCOSY, gHSQC and gHMBC) were used.

2.7. MS/MS measurements

MS/MS analysis of the protonated molecular ion of **A1** was performed on the ThermoQuest Finnigan LCQ Deca instrument using ESI in the positive ion mode under the following conditions: spray 3.0 kV, sheath gas 80 units, aux. gas 10 units, capillary voltage 20 V and capillary temperature 250 °C. The sample was injected as a 50 μg/ml solution in CH_3CN/H_2O 1/9 (v/v) mixture containing 1% HCOOH, by syringe pump injection into the ion source at a flow-rate of 20 μl/min. The MS/MS experiment was performed at a collision energy setting of 25 units.

2.8. Preparation of *A1*

To a suspension of (*1R,2S*)-2-amino-4-methylenecyclopentanecarboxylic acid [10] **1** (1.0 g; 7.08 mmol) and cinnamyl alcohol (1.74 g; 12.9 mmol) in EtOH (20 ml) were added Ph_3P (0.18 g, 0.69 mmol) and palladium(II)acetate (9.0 mg, 0.040 mmol) and the mixture was heated at 80 °C for 3 h. The mixture was cooled to r.t. and diisopropyl ether (18 ml) was added. The precipitated solid was collected by filtration, washed with diisopropyl ether (10 ml), placed in water (15 ml) and the pH was adjusted to 13.5 with 2% NaOH solution. After extraction with $CHCl_3$ (3×5 ml), pH of water layer was adjusted to 4.5. The resulted precipitate was collected by filtration and dissolved in MeOH (14 ml). To the clear solution diisopropyl ether (15 ml) was added and the mixture was stirred at r.t. for 20 h. The crystals were collected by filtration, washed with diisopropyl ether (8 ml) and dried in vacuo at 40 °C to afford **A1**, (1.16 g, 63.5%) as colourless crystals, mp 181–182 °C, $[\alpha]_D$ +52.1 (*c* 0.5, MeOH).

3. Results and discussion

3.1. LC–NMR and LC–MS analyses

For the successful separation of icofungipen **1** and the impurity **A1** the ratio of water and acetonitrile was varied until the best chromatographic peak separation was achieved (see Section 2). The retention times in the LC–DAD chromatogram were 2.2 min and 11.7 min for **1** and **A1**, respectively. Based on the area normalization in the LC–UV chromatogram the level of the impurity **A1** was estimated to be 1.2% in the row product. The prominent features of the DAD spectrum of the impurity were the presence of absorption maxima at 204 nm and at 252 nm. The absorption maximum for icofungipen was observed at 196 nm.

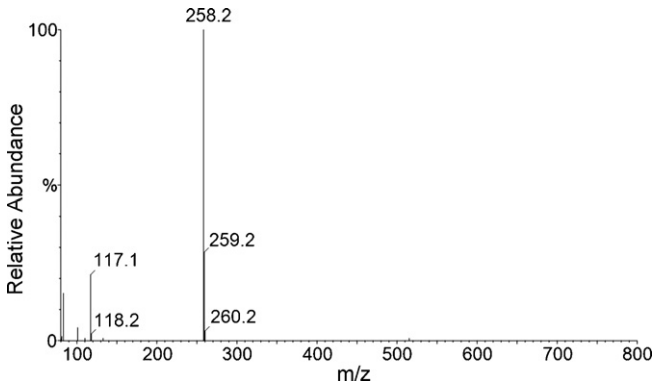


Fig. 1. LC–MS spectrum of the impurity **A1**.

The LC–MS spectrum of **A1** as shown in Fig. 1 exhibited a protonated molecular ion at *m*/*z* 258.2. The fragment ion at *m*/*z* 117.1 was also observed which is by 141 mass units lower than the precursor ion and corresponds to the mass of icofungipen. This was a strong indication that impurity **A1** was structurally closely related to icofungipen. The DAD spectrum and the observed fragmentation in the LC–MS spectrum indicated that **A1** might contain a cinnamyl moiety which could be anticipated from the fact that cinnamyl alchol was used in the preparation of icofungipen as described in the following section.

Subsequently, LC–NMR experiments were performed which provided further information about the structure of **A1**. The analysis of the ^{1}H LC–NMR spectrum revealed aliphatic and aromatic moieties (as shown in Fig. 2). The integration of the proton peak intensities gave a total number of non-exchangable protons which amounted to 17. Comparison between the ^{1}H LC–NMR spectra of **A1** and of icofungipen recorded in D_2O has indicated the presence of a cyclopentane ring and an additional side chain with aliphatic and aromatic structural units.

Two-dimensional DQCOSY LC–NMR spectrum was recorded next revealing some important structural connectivities. The correlation peaks in 2D COSY LC–NMR spectrum were diagnostic for the three spin-systems belonging to the benzene ring, methylenecyclopentane and –CH=CH–CH_2– moieties, respectively. The vicinal proton–proton coupling constant between the two olefinic protons (15.9 Hz) has pointed towards the *trans*-configuration of the double bond. Comparison of the coupling constants involving protons at the chiral centers of the cyclopentane ring in icofungipen and **A1** revealed similar configurations.

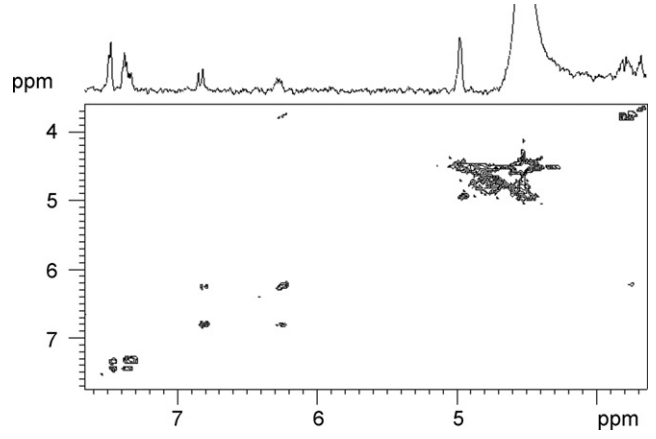
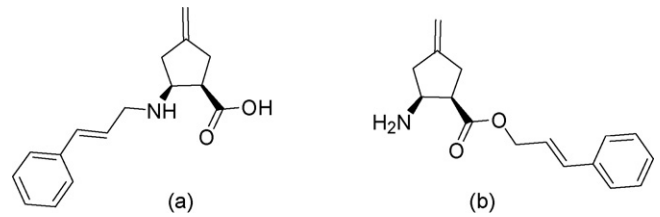


Fig. 2. The aromatic region of 2D DQFCOSY LC–NMR spectrum of **A1** showing the main correlation peaks.



Scheme 2. Structures of impurity **A1** (a) and its regioisomers (b).

Although complementary information obtained from the LC–MS and LC–NMR spectra have revealed several structural data on **A1**, the final chemical structure could not yet be deduced unambiguously. Namely, according to the available LC–UV, LC–NMR and LC–MS spectra the cinnamyl side-chain can be identified together with the methylenecyclopentane moiety but the position of the side-chain can be either at nitrogen atom thus forming an amine or at the carbonyl oxygen giving an ester (see Scheme 2). Although one can assume that the calculated chemical shift values for the methylene group at position 8 (Table 1) indicate an amine rather that an ester structure, we have undertaken further steps to resolve this ambiguity by employing tandem mass spectrometry (MS/MS). The recorded LC–MS/MS spectrum of **A1** exhibited the fragment ions at *m*/*z* 240.0 and 212.1 strongly indicating that the structure of impurity **A1** is the one designated as the structure **a** in Scheme 2. Namely, the fragment ion at *m*/*z* 240.0 pointed towards the loss of water molecule which would not be expected for the structure **b**. Also the ion at *m*/*z* 212.1 would only be possible if the structure **a** is the correct one. This is also in line with the chemical shift values. Therefore, we proposed the structure and fragmentation pattern of **A1** as depicted in Fig. 3 together with the direct infusion MS/MS spectrum of the isolated impurity **A1**.

3.2. Preparation of *A1*

Final steps in icofungipene synthesis [10] are outlined in Scheme 3. Monoester **2** was converted into the protected β-amino acid ester derivative **3** by Curtius rearrangement, followed by the reaction of the intermediate isocyanate with cinnamyl alcohol in refluxing toluene. In the last step, the protective groups in **3** were removed by transallylation with morpholine as nucleophile in refluxing ethanol, catalyzed with palladium acetate-triphenyl phosphine. The formation of **A1** can be explained by allylic amination of cinnamyl alcohol, delivered as an impurity from the previous step or liberated during the deprotection. Since, the deprotection reaction is a two-step process, both intermediates

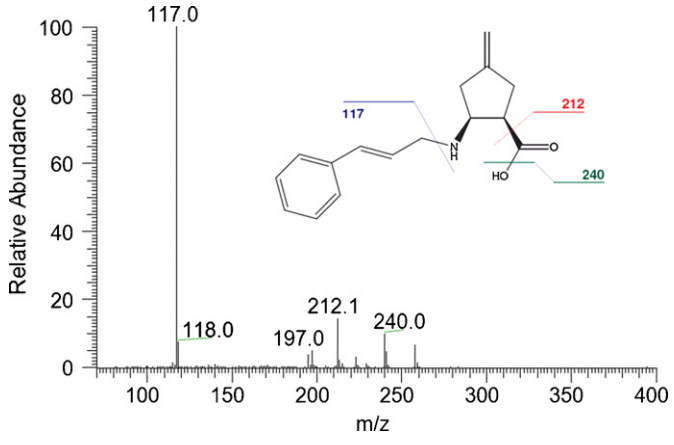
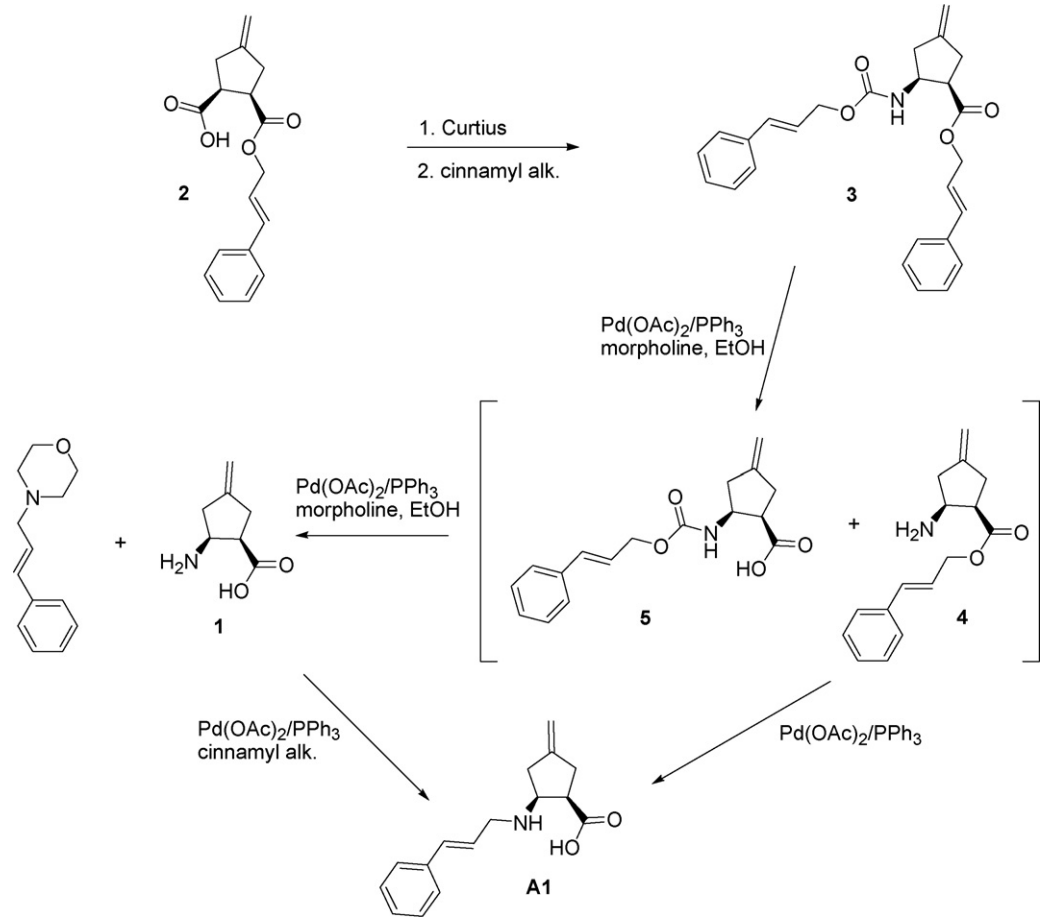


Fig. 3. MS/MS spectrum of **A1** and the fragmentation pathway.



Scheme 3. Synthesis of icofungipen (**1**) impurity **A1**.

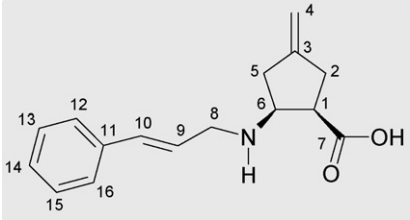
(**4** and **5**) being present in the reaction mixture, the possibility of intramolecular transallylation of intermediary **4** cannot be excluded. Presence of analogous impurities during the synthesis of icofungipene *endo*-isomers [11] and pregabaline [12], when the same methodology was employed, was not observed—presumably because of the different crystallization characteristics of substances.

(*1R,2S*)-2-(Cinnamylamino)-4-methylenecyclopentanecarboxylic acid **A1** was synthesized starting from (*1R,2S*)-2-amino-4-methylenecyclopentanecarboxylic acid **1** in Pd(II)-catalyzed reaction with cinnamyl alcohol in refluxing ethanol (Scheme 3). After usual work-up and recrystallization from MeOH/diisopropyl ether, pure product was isolated as a colourless crystalline substance in 63% yield.

3.3. Off-line NMR and MS/MS analyses

In order to confirm the proposed structure, impurity **A1** was isolated and prepared separately as described above and its structure was determined by high-resolution NMR spectroscopy and MS/MS spectrometry. A combination of one- and two-dimensional homo- and hetero-nuclear NMR experiments (APT, DQCOSY, HSQC and HMBC) led to the confirmation and full characterization of **A1**. Resonance assignment was made and proton and carbon chemical shifts are displayed in Table 1. The COSY spectrum revealed all the expected correlations as previously found in COSY LC–NMR spectrum. HSQC spectra yielded an unambiguous assignment for the protonated carbon atoms while correlation peaks in HMBC spectra revealed information about the quaternary carbons and confirmed the assignments of the protonated carbons. The HMBC correlation peaks confirmed the position of the side-chain at the methylenecyclopentane ring. The MS/MS spectrum showed the same fragmentation pattern (Fig. 3) as already observed in the LC–MS/MS spectrum.

Table 1
^{1}H and ^{13}C chemical shifts (ppm) of the icofungipen impurity **A1** in D_2O.



Atom	δ/ppm	
	^{1}H	^{13}C
1	3.08	46.35
2	2.66, 2.75	36.44
3	–	145.84
4	5.05	109.73
5	2.65, 2.79	35.35
6	3.79	58.99
7	–	180.88
8	3.87	48.84
9	6.34	119.52
10	6.90	139.00
11	–	136.45
12	7.56	127.79
13	7.45	129.91
14	7.40	129.85

In conclusion, a combination of LC–NMR and LC–MS was successfully used to characterize an icofungipene impurity prior to its isolation and purification steps.

Acknowledgement

We are indebted to Z. Kralj for his helpful comments and suggestions. The Ministry of Science, Education and Sports of the Republic of Croatia is acknowledged for the financial support (Project No. 119-1191342-1083.

References

[1] K. Ziegelbauer, P. Babczinski, W. Schönfeld, Antimicrob. Agents Chemother. 42 (1998) 2197–2205.
[2] J. Mittendorf, F. Kunisch, M. Matzke, H.C. Militzer, A. Schmidt, W. Schönfeld, Bioorg. Med. Chem. Lett. 13 (2003) 433–436.
[3] L.A. Sorbera, J. Castaner, J. Bozzo, Drugs Fut. 27 (2002) 1049–1055.
[4] R. Petraitiene, V. Petraitis, A.M. Kelaher, A.A. Sarafandi, D. Mickiene, A.H. Groll, T. Sein, J. Bacher, T.J. Walsh, Antimicrob. Agents Chemother. 49 (2005) 2084–2092.
[5] M.J. Parnham, J.J.P. Bogaards, F. Schrander, M.W. Schut, K. Orešković, B. Mildner, Biopharm. Drug Dispos. 26 (2005) 27–33.
[6] A. Hasenoehrl, T. Galić, G. Ergović, N. Maršić, M. Skerlev, J. Mittendorf, U. Geschke, A. Schmidt, W. Schönfeld, Antimicrob. Agents Chemother. 50 (2006) 3011–3018.
[7] P. Novak, P. Tepeš, M. Cindrić, M. Ilijaš, S. Dragojević, K. Mihaljević, J. Chromatogr. A 1033 (2004) 299–303.
[8] P. Novak, M. Cindrić, P. Tepeš, S. Dragojević, M. Ilijaš, K. Mihaljević, J. Sep. Sci. 28 (2005) 1442–1447.
[9] P. Novak, P. Tepeš, I. Fistrić, I. Bratoš, V. Gabelica, J. Pharm. Biomed. Anal. 40 (2006) 1268–1272.
[10] J. Mittendorf, J. Benet-Bucholz, P. Fey, K.-H. Mohrs, Synthesis (2003) 136–140.
[11] Z. Hameršak, M. Roje, A. Avdagić, V. Šunjić, Tetrahedron: Asymmetry 18 (2007) 635–644.
[12] Z. Hameršak, I. Stipetić, A. Avdagić, Tetrahedron: Asymmetry 18 (2007) 1481–1485.