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Isolation and characterization of process-related impurities in linezolid

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Abstract

Two unknown impurities in linezolid bulk drug at levels below 0.1% (ranging from 0.05 to 0.1%) were detected by a simple isocratic reverse phase high performance liquid chromatography (HPLC). These impurities were isolated from crude sample of linezolid using reverse phase preparative HPLC. Based on the spectroscopic data (IR, NMR and MS) the structures of the impurities were characterized as (*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] acetate(I) and (*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] chloride(II). The synthesis from an unambiguous route and the formation of impurities was discussed.

Keywords: Linezolid; Impurities; Spectroscopy; Identification and characterization

1. Introduction

The oxazolidinones are a new class of antimicrobials with good activity against gram positive bacteria [1,2]. Antimicrobiol resistance is a significant nosocomial problem and is of increasing importance in community-acquired infections. One approach for overcoming this resistance is the discovery and development of agents with a new mechanism of action. The oxazolidinones are a relatively new class of compounds which possess a unique mechanism of bacterial protein synthesis inhibition [3]. Linezolid, (*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]-methyl] acetamide, is a synthetic compound that acts by inhibiting the formation of initiation complex in bacterial protein synthesis, a mechanism of action which is distinct from that of any other antibiotics that are commercially available. Linezolid appeared to be an effective treatment option when compared to vancomycin [4,5]. Linezolid, administered orally [6], was launched in May 2000 for the treatment of patients with infections caused by gram positive bacteria [7]. A high

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performance liquid chromatographic method was cited in the literature for the assay of linezolid in plasma samples [8] using UV detection.

During the analysis of different batches of linezolid, two unknown impurities were detected whose area percentage ranged from 0.05 to 0.1%. A comprehensive study has been carried out to isolate and characterize these impurities. The stringent purity requirements from the customers that all the impurities $\geq 0.1\%$ must be identified and characterized. This paper aims at the isolation and characterization of two prominent impurities that are present at a level of $\leq 0.1\%$ in the bulk drug of linezolid. An in-house LC method was developed and validated [9] for the analysis of linezolid and its intermediates, where a C18 column with a mobile phase of water and acetonitrile was used with a UV detection at 250 nm. This LC method was able to detect these impurities which ranged from 0.05 to 0.1% in the presence of parent compound.

2. Experimental

2.1. Samples

The investigated samples of linezolid bulk material (B.No. LIN-50) and crude samples (B.No. LIN-mother liquors) were obtained from Process Research and Technology Development Department of Dr. Reddy's Research Foundation, Hyderabad, India.

2.2. High performance liquid chromatography (Analytical)

A Waters Model Alliance 2690 Separation module equipped with a Waters 996 photo diode array UV detector was used. An Inertsil ODS-3V column with the dimensions of 250 × 4.6 mm i.d. (GL Sciences, Japan) was used for the separations. The column eluent was monitored at a wavelength of 250 nm and the data was recorded using Waters Millennium 32 software. A mixture of water and acetonitrile in the ratio of 65:35 (v/v) was used as mobile phase at a flow rate of 1.0 ml/min.

2.3. High performance liquid chromatography (Preparative)

A Shimadzu preparative HPLC equipped with LC-8A pump, SCL-8A System controller, SPD-6AV UV–VIS detector, FCV-100B Fraction collector and Rheodyne Injector Model 7725i with 2.0 ml loop. The data was collected and processed using Shimadzu CR7A chromatopak. A 250 × 10 mm i.d. column packed with 5 μ Hichrom-C18 (Hichrom Ltd., UK) was employed for separation. The mobile phase consisted of water–acetonitrile in a ratio of 65:35 (v/v). The flow rate was set at 2.0 ml/min. Detection was carried out at 250 nm.

2.4. Mass spectrometry

Mass spectra were run on HP5989 with ionisation electron beam energy of 70 eV. The sample was introduced into the source with the help of a particle beam interface connected to LC by bypassing the column. The source manifold and Quadrupole temperatures were maintained at 250 and 100 °C, respectively. The CI reagent gas used was isobutane.

2.5. NMR spectroscopy

NMR measurements were performed on a Varian Gemini 2000 model 200 MHz instrument (both for ^{1}H and ^{13}C) at 25 °C in $CDCl_3$.

The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm, relative to TMS ($\delta = 0.00$) and $CDCl_3$ ($\delta = 77.0$ ppm) as internal standard, respectively.

2.6. FT-IR spectroscopy

The IR spectra for linezolid, impurity I and II were recorded in the solid state as KBr dispersion using Perkin–Elmer 1650 FT-IR spectrophotometer.

2.7. Synthesis of impurities

2.7.1. Synthesis of impurity I

(*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-5-hydroxymethyl-2-oxazolidinone(III) was taken in ethyl acetate and cooled to 5–10 °C. Triethyl amine was added to the reactants. Acetic anhydride was added slowly at 5–10 °C for 5 min. The reaction mixture was brought to room temperature and stirred for 6–8 h [10]. The reaction progress was monitored by thin layer chromatography (TLC) using ethyl acetate and petroleum ether in 2:1 v/v ratio. After the completion of the reaction, the reaction mixture was stirred in petroleum ether for 10 min and filtered. The solid obtained was washed with petroleum ether and dried. The intermediate (III) was converted into impurity I.

IR data of impurity I (*all frequencies are in* cm^{-1}): 1748 (C=O and Lactone stretching), 1526 (aromatic C=C stretching), 1229 and 1198, (C–O stretching), 1115 (C–F stretching).

MS data of impurity I: m/z (EI; rel. int.,%) 280 (14), 234 (17), 208 (17), 176 (36), 164 (23), 150 (72), 136 (13), 122 (13), M + 338 (100).

2.7.2. Synthesis of impurity II

(*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-5-hydroxymethyl-2-oxazolidinone(III) was allowed to react with thionyl chloride [11]. Few drops of dimethylformamide were added to the reaction mixture to facilitate fast reaction. The reaction mixture was stirred at room temperature for 10 h. The reaction progress was monitored by TLC using ethyl acetate and petroleum ether in 2:1 v/v ratio. After completion of the reaction, the reaction mixture was poured on ice and stirred for 10 min. The compound was extracted into ethyl acetate and washed with DM water (de-mineralized water) and the organic layer was concentrated. The intermediate (III) was converted into impurity II.

IR data of impurity II (*all frequencies are in* cm^{-1}): 1756 (lactone stretching), 1517 (aromatic C=C stretching), 1230 (C–O stretching), 1116 (C–F stretching), 733 (C–Cl stretching).

MS data of impurity II: m/z (EI; rel. int.,%) 256 (660, 177 (63), 149 (79), 122 (21), M + 314 (100), M + 2316 (37 chlorine isotopic abundance).

IR data of linezolid (*all frequencies are in* cm^{-1}): 3343 (N–H stretching), 1740 (lactone stretching), 1662 (amide I stretching), 1547 (aromatic C=C stretching), 1517 (amide II stretching), 1230 (C–O stretching), 1118 (C–F stretching).

MS data of linezolid: m/z (EI; rel. int.,%) 293 (67), 234 (58), 222 (15), 209 (75), 176 (48), 164 (40), 151 (40), 139 (38), 122 (15), M + 337 (100).

3. Results and discussions

3.1. Detection of impurities I and II

A typical analytical LC chromatogram (Fig. 1a) of a laboratory batch of linezolid bulk drug recorded using the LC method as described in Section 2.2. The target impurities under study are marked as Imp-I and Imp-II which eluted at retention times of about 15 and 22 min, respectively, while linezolid eluted at about 5 min. These impurities were isolated from the mother liquor sample of linezolid on preparative LC for spectroscopic studies.

3.2. Isolation of the impurities by preparative HPLC

A simple reverse phase solvent system discussed under Section 2.3 was used for isolating these impurities. In this solvent system linezolid eluted at about 12–14 min whereas the impurities I and II eluted at about 18–20 and 30–35 min, respectively. Collected fractions of these impurities were pooled together and kept in the refrigerator. Both the fractions of impurities isolated were concentrated under high vacuum on a Buchii Rotavapor Model R124. Purity of these impurities was tested before and after concentration in analytical mode (Section 2.2) and found to be 94 and 98%, respectively which shows that these impurity fractions are quite stable during and after isolation. The concentrated fractions of impurities were used to generate spectral data.

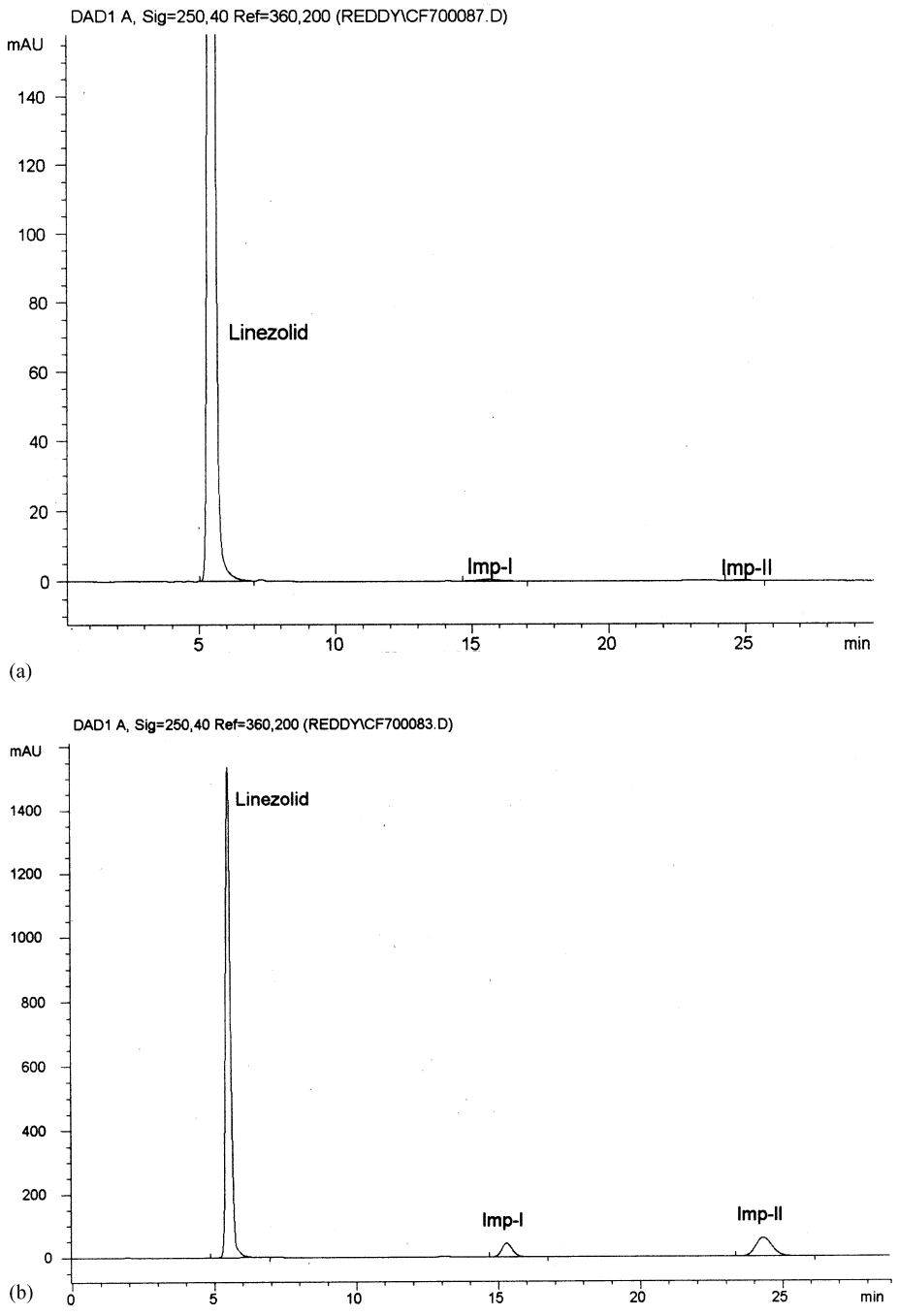


Fig. 1. LC Chromatograms of linezolid (a) and spiked chromatogram of linezolid with impurities (b).

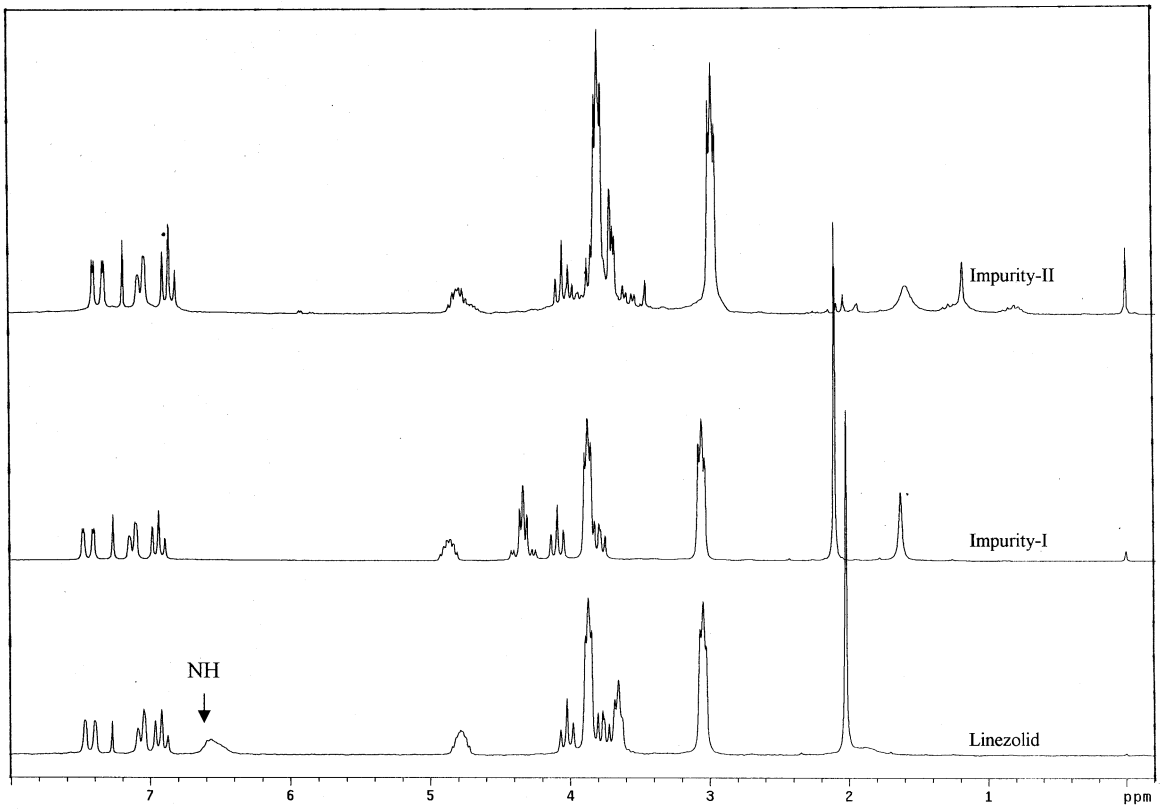


Fig. 2. Overlaid ^{1}H NMR spectra of linezolid, impurities I and II.

3.3. Structural elucidation of impurities I and II

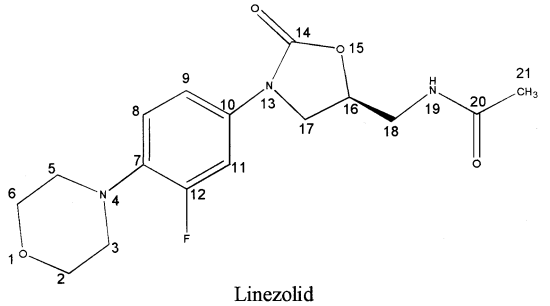
The spectral data of impurities I and II was compared with those of linezolid. The FT-IR spectrum of linezolid exhibited a characteristic stretching absorption band between 3380 and 3400 cm^{-1} indicating the presence of NH group. These bands were absent in the IR spectra of both impurities I and II. Further, the strong C=O stretching (amide band-I) and bending which results from the interaction between N–H bending and C–N stretching (amide band-II) bands at 1662 and 1517 cm^{-1}, respectively in linezolid were absent in the FT-IR spectra of both impurities I and II. The disappearance of these characteristic bands due to amide indicate the absence of NH group in impurities I and II. Further an exchangeable proton signal for NH at δ 6.2 ppm in linezolid was absent in the ^{1}H NMR spectra of impurities I and II as shown in Fig. 2. These observations clearly demonstrate the absence of –NH (present in linezolid) in both the impurities.

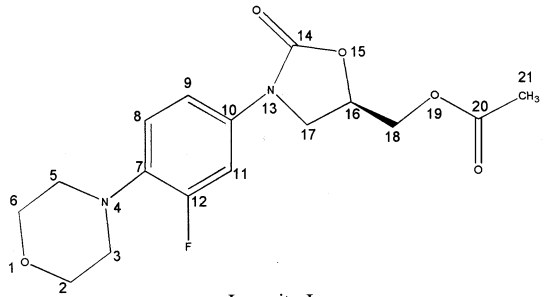
The CI mass spectrum of impurity I displayed a protonated molecular ion peak at m/z 339 which is 1 atomic mass unit (amu) more than that of linezolid which indicates the possible incorporation of oxygen in place of NH group in linezolid. The corresponding molecular formula of the impurity could be $C_{16}H_{19}FN_2O_5$.

The CI mass spectrum of impurity II displayed a protonated molecular ion at m/z 315 which is 23 amu less than that of linezolid. The CI mass spectrum also exhibited (M + 2) molecular ion in 3:1 ratio which is characteristic of the presence of chlorine in impurity II and the corresponding molecular formula could be $C_{14}H_{16}ClFN_2O_3$. Further, the FT-IR spectrum of impurity II also displayed a strong characteristic C–Cl stretching absorption band at 733 cm^{-1}.

The ^{1}H and ^{13}C NMR chemical shift values for linezolid, impurities I and II are given in Table 1. The structures of impurities I and II are characterized as (*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] acetate(I) and (*S*)-*N*-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] chloride(II), respectively are shown in Fig. 3.

The spectral data synthesized impurities were generated and were found to match with those of isolated impurities. The synthetic standards of impurities I and II were co-injected on LC with linezolid and the area percentage at retention times 15 and 22 min were enhanced and the LC chromatogram is shown in Fig. 1b.





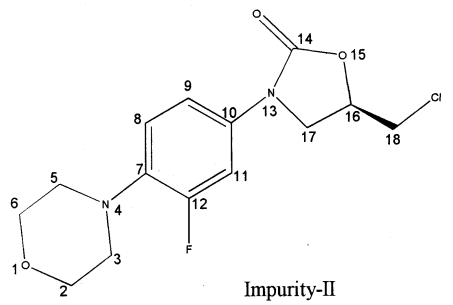


Fig. 3. Chemical structures of linezolid, impurities I and II.

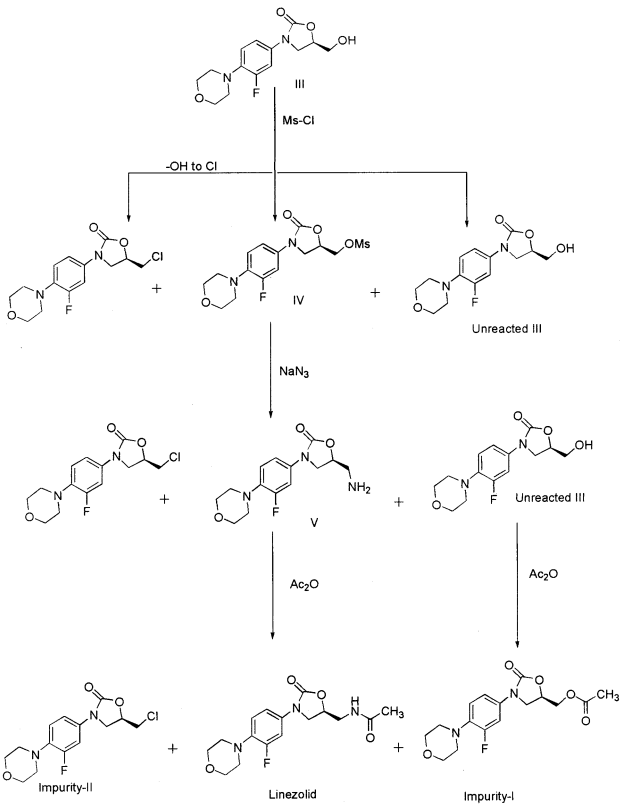


Fig. 4. Scheme for the formation of impurities I and II.

3.4. Formation of impurities

3.4.1. Formation of impurity II

The key intermediate in the synthesis of linezolid is III. This (III) is converted to its corresponding mesyl derivative (IV) using mesyl chloride. During this reaction, the hydroxy group in III might be replaced by chlorine in trace levels leading to the formation of impurity II.

3.4.2. Formation of impurity I

During the reaction of III with mesyl chloride which lead to the formation of its corresponding mesyl derivative (IV), some amount of unreacted III may also be present in IV. In the next step of the synthesis of linezolid, the mesyl derivative (IV) is converted to its corresponding amine derivative (V) using sodium azide. During this reaction also the unreacted III might be present in V. The amine derivative (V) upon reaction with acetic

Table 1
^{1}H and ^{13}C NMR data of linezolid, impurities I and II

Position	Linezolid			Impurity-I			Impurity-II		
	^{1}H	ppm/J	^{13}C	^{1}H	ppm/J	^{13}C	^{1}H	ppm/J	^{13}C
2 and 6	4H	3.8/t (4.5)	66.5	4H	3.8/t (4.5)	66.7	4H	3.7/t (4.4)	66.6
3 and 5	4H	3.1/t (4.5)	50.6	4H	3.2/t (4.5)	50.8	4H	3.0/t (4.4)	50.8
7	–	–	132.6, (10.2[a])	–	–	132.8, (10.2[a])	–	–	132.8 (10.2[a])
8	1H	6.9/t (9.0)	118.4, (4.1[a])	1H	6.9/t (9.0)	118.6, (4.2[a])	1H	6.9/t (9.1)	118.7 (4.5[a])
9	1H	7.1/dd (2.5, 8.8[b])	113.6, (3.5[a])	1H	7.1/dd (2.5, 8.8[b])	113.6, (3.4[a])	1H	7.1/dd (2.5, 8.8[b])	113.7 (3.5[a])
10	–	–	136.0, (8.8[a])	–	–	136.3, (9.2[a])	–	–	136.5 (9.3[a])
11	1H	7.5/dd (2.5, 14.5[b])	107.5, (25.7[a])	1H	7.5/dd (2.5, 14.5[b])	107.3, (26.2[a])	1H	7.5/dd (2.5, 14.5[b])	107.4 (26.2[a])
12	–	–	154.9, (245[a])	–	–	155.2, (245[a])	–	–	155.3 (245[a])
14	–	–	153.4	–	–	153.9	–	–	153.7
16	1H	4.8/m	71.8	1H	4.8/m	69.8	1H	4.8/m	70.7
17	2H	4.0/t (9.0)	47.3	2H	4.1/t(9.0)	46.9	2H	4.1/t(8.8)	47.9
18	2H	3.7/m	41.5	2H	4.4/d(4.4)	63.9	2H	3.7/t(7.4)	44.6
19	NH	6.2/m	–	–	–	–	–	–	–
20	–	–	171.2	–	–	170.4	–	–	–
21	3H	2.0/s	22.6	3H	2.1/s	20.5	–	–	–

Refer structural formula for numbering (Fig. 3). s, singlet; d, doublet; m, multiplet; dd, doublet of doublet; J-coupling constant.

[a] ^{13}C–^{19}F coupling constant.

[b] ^{1}H–^{19}F coupling constant.

anhydride in the presence of a base is converted to linezolid. During the process of formation of linezolid, the unreacted III might also undergo acetylation leading to the formation of impurity I.

The scheme for the formation of impurities I and II is shown in Fig. 4.

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