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SYNTHESIS AND CHARACTERIZATION OF DEGRADATION IMPURITIES OF AN ANTIBIOTIC DRUG: LINEZOLID

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ABSTRACT: First direct synthesis of typical Linezolid degradation impurities (*R*)-N-(3-(3-fluoro-4-morpholinophenylamino)-2-hydroxypropyl) acetamide and (*S*)-1-amino-3-(3-fluoro-4-morpho linophenylamino) propan-2-yl acetate were reported. (*R*)-N-(3-(3-fluoro-4-morpholinophenyl amino)-2-hydroxypropyl) acetamide was synthesized starting from3-fluoro-4-morpholinylaniline with epichlorohydrin following 4 steps. (*S*)-1-amino-3-(3-fluoro-4-morpholinophenylamino) propan-2-yl acetate was synthesized starting from 3-fluoro-4-morpholinyl aniline with epichloro hydrin following 6 steps. Along with the above impurities three more process impurities also synthesized. All the synthesized compounds were confirmed by their ^{1}H, ^{13}C NMR and Mass spectral analysis. The two degradation impurities can be used as a reference substance in the quality checking of Linezolid injection.

KEYWORDS: Linezolid, Degradation products, Impurities, Synthesis

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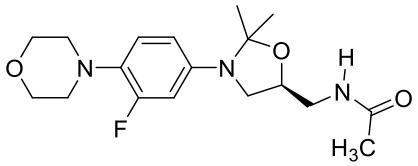
1.INTRODUCTION

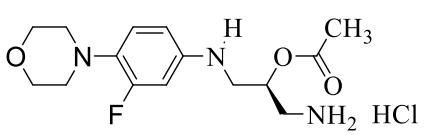
Oxazolidinones were identified as key structural core units, which exhibit good activity against gram positive bacteria [1-4]. Linezolid (1) (Figure 1) which was discovered in the mid of 1990s and was approved for commercial use in 2000 against all of the major gram-positive bacteria that are pathogenic to humans [5, 6].



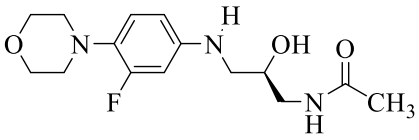
Linezolid (1)

Figure 1. Linezolid

Development of degradation impurities is less explored which often occurs during the preparation of injections and solid oral dosages. In the development of Linezolid injection few degradation impurities were identified which were formed due to high-temperature sterilization and long-term placement process. Since the formation of impurities will decrease the quality of the injection interns affect drug safety and efficacy of clinical use. Due to our continuous interest towards the development of heterocyclic active core **4** was chosen to synthesize [7] the degradation impurities which were identified during the preparation of Linezolid injections. Here in, we report the synthesis of two main degradation impurities (*S*)-1-amino-3-(3-fluoro-4-morpholinophenyl amino) propan-2-yl acetate (**2**) and (*R*)-N-(3-(3-fluoro-4-morpholinophenylamino)-2-hydroxypropyl) acetamide (**3**) and three process related impurities (**6**, **8**, **9**) also synthesized.



Linezolid related impurity (2)



Linezolid related impurity PNU140155 (3)

Figure 2. Degradation impurities of Linezolid

The chemical structures of the two impurities were shown in figure 2. The synthesis of impurities were reported in the literature [8-10], but the process was having low yield with several steps [11]. In this paper we underwent to the synthesis of two important degradation impurities along with other three process related impurities.

2. MATERIALS AND METHODS

Unless stated otherwise, reactions were monitored by thin layer chromatography (TLC) on silica gel plates (60 F254), visualizing with ultraviolet light and Ninhydrine spray. Column chromatography was performed on silica gel (100-200 mesh) using distilled petroleum ether and ethyl acetate. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$ and DMSO-d_6 solutions using 400 MHz and 100

MHz spectrometers, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, δ = 0.0) as internal standard and expressed in parts per million. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), m (multiplet) and bs (brod singlet). Coupling constants (*J*) are given in hertz (Hz). Mass analysis was done on ACQUITY QDa Mass Detector.

Synthesis of (S)-1-amino-3-(3-fluoro-4-morpholinophenylamino)propan-2-ol (9): To stirred solution of compound **8** (14 g, 35.09 mmol.) in MeOH (140 mL, 10 Vol.) was added 75% Hydrazine hydrate (9.08 g, 283.3 mmol.) at room température under nitrogen atmosphere. The reaction was heated to reflux for 3 h. After completion of the reaction (TLC), all the solvents were evaporated under redused pressure to get crude compound. The crude compound was stirred with EtOAc (100 mL), filtered and washed with EtOAc (25 mL). The filtrate was distilled under redused pressure to obtaind compound **9** as a brown solid (8.5 g, yield 90.4%). Rf 0.15 (1 :1, EtOAc-IPA); ^{1}H NMR (DMSO-d_6, 400 MHz); δ 1.36 (bs, 2H), 2.49 (m, 1H), 2.56-2.60 (dd, 1H, J_1=4.64 Hz, J_2=12.72 Hz), 2.79-2.81 (t, 4H, *J*=4.3 Hz), 2.83-2.86 (m, 1H), 2.99-3.05 (m, 1H), 3.47-3.3.49 (m, 1H), 3.66-3.69 (t, 4H, *J*=4.2 Hz), 4.71 (bs, 1H), 5.43-5.46 (t, 1H, *J*=5.64 Hz), 6.32-6.34 (dd, 1H, J_1=2.0 Hz, J_2=8.64 Hz), 6.38-6.42 (dd, 1H, J_1=2.12 Hz, J_2=15.24 Hz) and 6.78-6.83 (t, 1H, *J*=9.32 Hz) ; ^{13}C NMR (DMSO-d_6 100 MHz); δ 157.7, 155.3, 146.1, 146.0, 128.8, 128.7, 120.5, 107.7, 100.3, 100.0, 70.7, 66.4, 51.6, 47.4 and 46.0; Mass m/z; 270.1 $(M+H)^+$.

Synthesis of (R)-*N*-(3-(3-fluoro-4-morpholinophenylamino) -2-hydroxypropyl)acetamide (3): To stirred solution of compound **9** (12 g, 44.57 mmol.) in EtOAc (120 mL, 10 Vol.) and TEA (10 mL, 0.83 Vol.) was added Ac_2O (6.5 g, 63.73 mmol.) at 0-5 ^{o}C under nitrogen atmosphere. The reaction mixture was stirred at 0-5 ^{o}C for 30 min and then warmed to RT and stirred for 1 h. After completion of the reaction (TLC), the reaction mixture was filtered and washed with EtOAc (25 mL). The filtrate was distilled under redused pressure to get crude compound as a brown gummy solid. The crude compound was dissolved in a mixture of EtOAc (100 mL) and water (50 mL) then stirred for 30 minutes. The two layers were separates and the aqueous layer was extracted with EtOAc (50 mL x 3) and the combined organic layers (3 extractions) was dried over anhy. Na_2SO_4. The organic layer was evaporated under redused pressure to obtained compound **3** (Linezolid impurity PNU140155) as a white solid (10.53 g, yield 75.91%). Rf 0.50 (8 :2, EtOAc-IPA); ^{1}H NMR (DMSO-d_6, 400 HHz): δ. 1.82 (s, 3H, CH_3), 2.81-2.89 (m, 5H), 2.97-3.07 (m, 2H), 3.13-3.19 (m, 1H), 3.59-3.65 (m, 1H), 3.68-3.70 (t, 4H, *J*=4.24 Hz), 4.95-4.97 (d, 1H, *J*=4.92 Hz), 5.45-5.48 (t, 1H, *J*=5.8 Hz), 6.32-6.43 (m, 2H, Ar-H), 6.80-6.85 (t, 1H, *J*=9.16 Hz, Ar-H) and 7.84-7.87 (t, 1H, *J*=5.4 Hz, NH); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 169.8, 157.9, 146.1, 129.2, 120.7, 108.0, 100.6, 68.2, 66.6, 51.8, 47.7, 43.4, 22.7; Mass m/z: 312.2 $(M+H)^+$.

Synthesis of Tri Boc Compound (10): To stirred solution of compound **9** (3.0 g, 11.14 mmol.) in

THF (30 mL, 10 Vol.) was added $(Boc)_2O$ (14.6 g, 66.84 mmol.) and DMAP (13.58 g, 111.14 mmol) at room temperature under nitrogen atmosphere. The reaction mixture was heated to reflux and continued for another 5 h. After completion of the reaction (TLC), the reaction was cooled to room temperature, water (100 mL) was added and then extracted with n-hexane (50 mL x 3). The combined organic layers were dried over anhy. Na_2SO_4 and evaporated under redused pressure to get crude product. The crude compound was purified by column chromatography using silica gel (20% EtOAc in Hexane) to obtaind compound **10** as off-white solid (4.82 g, Yield 76.51%). Rf 0.85 (1:1, EtOAc-hexane); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.47 (s, 9H), 1.51 (s, 18H), 2.95-2.97 (t, 4H, *J*=4.8 Hz), 3.26-3.28 (m, 1H), 3.84-3.86 (t, 4H, *J*=4.8 Hz), 3.90-3.91 (d, 2H, *J*=5.6 Hz), 4.10 (bs, 1H), 4.97-4.5.03 (m, 1H), 6.34-6.40 (m, 2H) and 6.79-6.84 (t, 1H, *J*=8.8 Hz); ^{1}H NMR (DMSO-d_6 400 MHz); δ 1.35 (s, 9H), 1.41 (s, 18H), 2.78-2.82 (t, 4H, *J*=4.8 Hz), 3.08-3.20 (m, 2H), 3.65-3.77 (m, 6H), 4.93-4.94 (d, 1H, *J*=4.4 Hz), 5.76-5.79 (m, 1H), 6.33-6.42 (m, 2H) and 6.79-6.84 (t, 1H, *J*=9.2 Hz); ^{13}C NMR (DMSO-d_6 100 MHz); δ 157.61, 155.20, 152.46, 151.90, 145.26, 145.16, 129.24, 129.15, 120.51, 107.70, 100.25, 100.01, 81.78, 81.16, 73.18, 66.33, 51.51, 46.77, 44.34, 27.47 and 27.22; Mass m/z; 570.4 $(M+H)^+$.

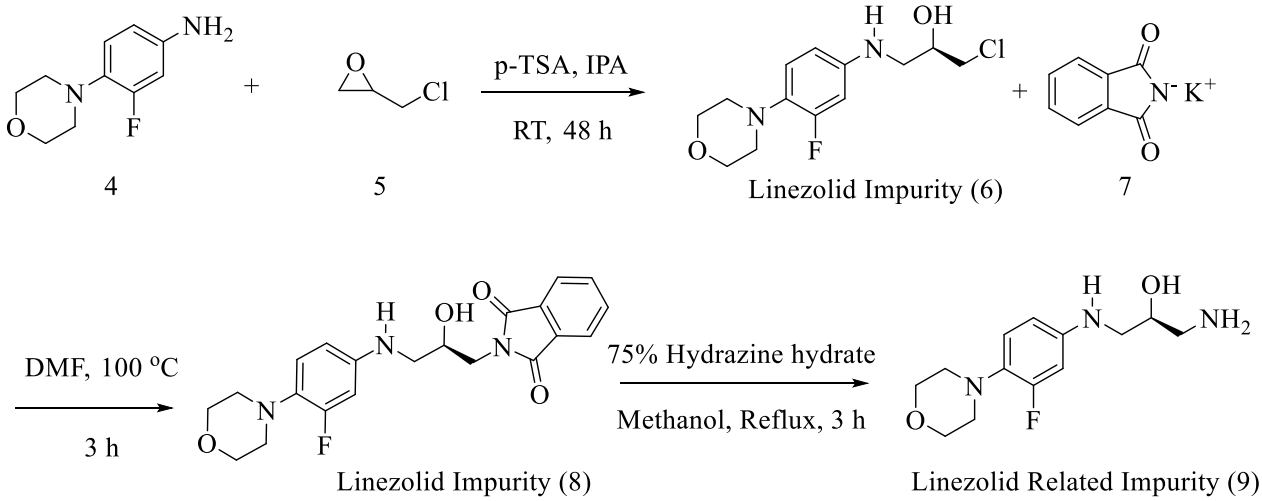
Synthesis of Tri Boc with *O*-Acetyl Compound (11) : To a solution of compound **10** (2.0 g, 3.51 mmol.) in DMF (20 mL, 10 vol.) was added DMAP (1.3 g, 10.53 mmol.) and acetyl chloride (1.1 g, 14.04 mmol.) at room temperature under nitrogen atmosphere. The reaction mixture was maintained 2 h. After completion of the reaction (TLC), water (50 mL) was added to the reaction mixture, extracted with MTBE (50 mL x 3) and washed with water (50 mL x 2) followed by dried over anhy. Na_2SO_4. The solvent was removed under redused pressure to get crude product which was purified by column chromatography using silica gel (30% EtOAc in Hexane) to get compound **11** as off-white solide (1.5 g, yield 70.1%). Rf 0.50 (1:1, EtOAc-hexane); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 9H), 1.50 (s, 18H), 1.84 (s, 3H), 3.09-3.12 (t, 4H, *J*=4.8 Hz), 3.72-3.81 (m, 2H), 3.87-3.93 (m, 6H), 5.08-5.12 (m, 1H), 6.88-6.92 (t, 1H, *J*=8.8 Hz) and 6.96-7.02 (m, 2H); ^{1}H NMR (DMSO-d_6, 400 MHz); δ 1.35 (s, 9H), 1.40 (s, 18H), 1.73 (s, 3H), 3.01 (bs, 4H), 3.54-3.58 (m, 1H), 3.63-3.78 (m, 7H), 4.97 (m, 1H) and 7.04-7.14 (m, 3H); ^{13}C NMR (DMSO-d_6 100 MHz); δ 169.58, 155.40, 152.95, 152.32, 151.69, 138.99, 136.97, 136.87, 124.32, 119.08, 116.05, 115.84, 81.86, 81.25, 72.88, 66.07, 50.29, 49.52, 46.62, 27.46, 27.40 and 22.28: Mass m/z: 612.4 $(M+H)^+$.

Synthesis of (S)-1-amino-3-(3-fluoro-4-morpholinophenylamino)propan-2-ylacetate hydrochloride (2): To a solution of compound **11** (3.0 g, 4.91 mmol.) in 1,4-Dioxane (15 mL, 5.0 Vol.) was added 4.0 M HCl in 1,4-Dioxane (15 mL, 5 Vol.) at room temperature under nitrogen atmosphere. The reaction mixture was stirred for 2 h. After completion of the reaction (TLC), MTBE (100 mL) was added then stirred for 30 min and filtered. The solid compound was dried under redused pressure to obtained compound **2** (Linezolid related impurity 2) as yellow solid (1.32 g,

yield 77.62%). Rf 0.30 (2:8, MeOH-DCM); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.83 (s, 3H, CH_3), 3.08 (s, 5H), 3.29 (bs, 1H), 3.75 (bs, 2H), 3.85 (s, 4H), 4.28 (bs, 1H), 6.92-6.96 (t, 1H, *J*=8.76 Hz), 7.13-7.16 (m, 2H) and 7.98 (bs, 3H); ^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm**:** 1.75 (s, 3H), 2.63-2.67 (m, 1H), 2.90-2.92 (m, 1H), 3.02 (s, 4H), 3.41-3.46 (m, 1H), 3.72-3.80 (m, 6H), 5.51 (bs, 1H), 7.03-7.08 (t, 1H, *J*=9.3 Hz), 7.16-7.18 (d, 1H, *J*=8.2 Hz), 7.30-7.33 (d, 1H, *J*=13.7 Hz) and 7.87 (bs, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.4, 160.6, 139.4, 133.9, 122.4, 118.7, 104.6, 72.2, 66.7, 47.3, 45.9, 21.2 ppm; Mass m/z: 312.3 $(M+H)^+$.

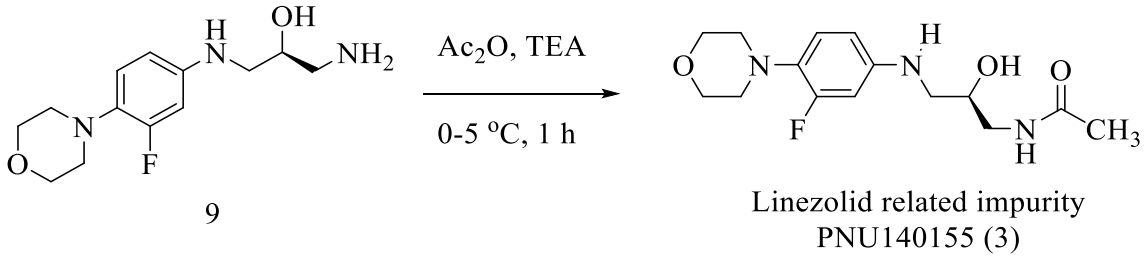
3. RESULTS AND DISCUSSION

During the development of Linezolid injection, we observed two major degradation impurities ranging from 0.01 to 0.15%, when injected the testing sample in HPLC analysis. According to ICH (International Conference on Harmonization) guidelines the acceptable level for known and unknown impurities in a final drug candidate must be less than 0.15% and 0.10% respectively. To control the formation of the degradants formed during the preparation of Linezolid injections the two impurities needed to be synthesized and characterized. During the synthesis we also prepared three more process related Linezolid impurities. The assigned structure of these degradation impurities is found to be compound **2** and **3**. Along with the above two impurities we also synthesized the impurity **6**, impurity **8** and related impurity **9** which are formed during the process. Linezolid Impurity **4**, a potential impurity formed during the synthesis of Linezolid. 3-fluoro-4-morpholinoaniline **(4)** treated with epichlorohydrin (**5)** in IPA by using p-TSA to form compound **6**. process related impurity **8** was synthesized from Compound **6** on heating with potassium pthalamide (**7**) in dimethyl formamide at 100 ^oC for 3 h yielded compound **8.** Compound **9** was synthesized by the deprotection of compound **8** with 75% hydrazine hydrate in methanol at reflux (Scheme 1) [12-15].



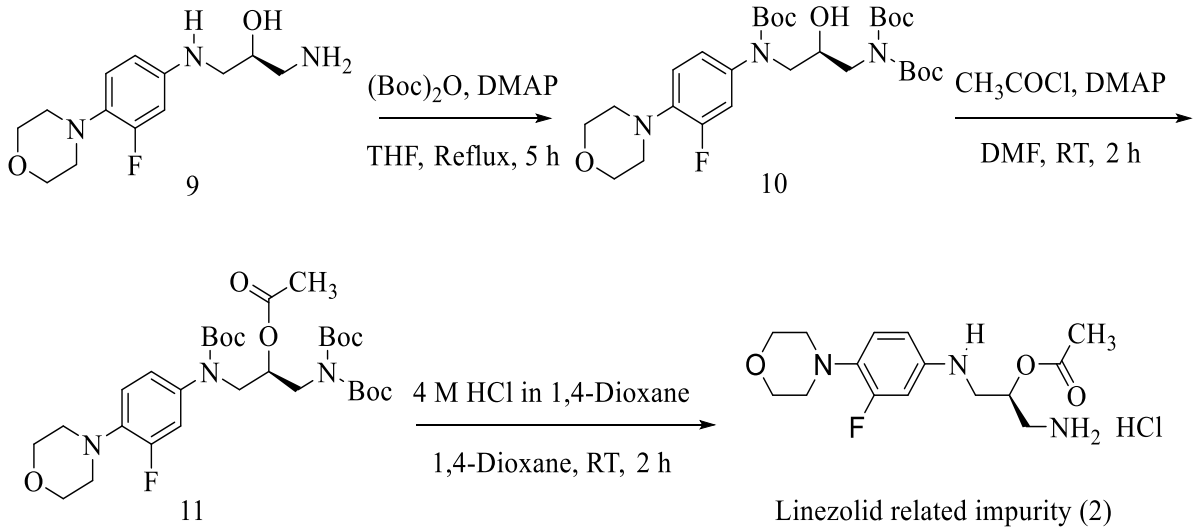
Scheme 1. Synthesis of Linezolid Impurities (**6**, **8** & **9**)

Compound **3**, which is a potential impurity formed during the degradation of linezolid (**1**). This impurity was synthesized by acetylation of compound **9** using acetic anhydride in ethyl acetate at 0-5 ^{o}C under mild conditions and its structure was further confirmed by ^{1}H, ^{13}C NMR and Mass (Scheme 2) [16-18].



Scheme 2. Synthesis of Linezolid Impurity (3)

Linezolid related impurity **2** which is another potential degradation impurity observed during the preparation of Linezolid injection was synthesized from compound **9**. Compound **9** which on reaction with Boc anhydride in THF at room temperature yielded tri Boc compound **10** as off-white solid [19, 20]. Compound **10** is on acylation with acetyl chloride using DMAP in DMF yielded compound **11** as off-white solid [21]. Compound **11** on Boc deprotection with 4 M HCl in dioxane in 1,4-Dioxane at room temperature for 2 h yielded Linezolid related impurity **2** was further confirmed by ^{1}H, ^{13}C NMR and Mass (Scheme 3) [22].



Scheme 3. Synthesis of Linezolid Impurity (**2**)

All the synthesized compound were thoroughly analyzed using ^{1}H, ^{13}C NMR and mass spectral data followed by confirming the RT and RRT using HPLC method.

4. CONCLUSION

In conclusion, we have synthesized and characterized two degradation impurities and three process impurities of Linezolid (**1**). The structures of the above compounds were confirmed using spectral analysis, and the purity was determined by HPLC.

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