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Synthesis and Characterization of Key Stereoisomers Related to Eluxadoline

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Eluxadoline is a novel active pharmaceutical ingredient (API) employed in the treatment of diarrhea and abdominal discomfort associated with diarrhea-predominant irritable bowel syndrome (IBS-D). Throughout the laboratory optimization and late-phase manufacturing studies of eluxadoline, the emergence of several stereoisomers was observed. To elucidate the comprehensive stereoisomer profile of eluxadoline, we have synthesized and meticulously characterized the (*R*,*R*)-eluxadoline enantiomer impurity, (*S*,*R*)-eluxadoline diastereomer impurity and (*R*,*S*)-eluxadoline stereoisomers of eluxadoline for the first time. These impurities were identified, synthesized and characterized with IR, ^{1}H NMR, ^{13}C NMR and mass spectra. This investigation will facilitate access to reference standards of these stereoisomers and may bear significant implications for the advancement of new medicinal therapies.

Keywords: Eluxadoline, Synthesis, Characterization, Stereoisomeric impurities.

INTRODUCTION

Eluxadoline or 5-({[(2*S*)-2-amino-3-(4-carbamoyl-2,6-dimethylphenyl)propanoyl][(1*S*)-1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid (Fig. 1) is a mixed opioid receptor agonist and antagonist, acting as an agonist for the mu (μ) receptor and an antagonist for the delta (δ) receptor [1]. Eluxadoline's multimodal opioid pharmacology appears to enable it to efficiently relieve abdominal pain and bowel movements in IBS-D patients while limiting the risk of constipation. It acts locally in the gastrointestinal (GI) tract, helping to relieve abdominal pain and regulate bowel movements while minimizing constipation. Eluxadoline is marketed as Viberzi in the US and Truberzi in Europe [2]. It is an oral drug for treating diarrhea and abdominal pain in diarrhea-predominant irritable bowel syndrome (IBS-D) [3], approved in the USA in 2015 [4]. Eluxadoline's positive benefits in treating diarrhea predominant irritable bowel syndrome (IBS-D) are due to its local action within the GI tract, where opioid receptors are widely expressed and play a crucial role in regulating GI motility, secretion and visceral sensation [5-7].

Several clinical studies showed that eluxadoline is effective in the treatment of severe irritable bowel syndrome with diarrhea (IBS-D) in adult men and women [8-10]. Identification and control of impurities in drug compounds and drug products are crucial and critical for their safety assessment. These impurities affect the drug's efficacy, efficiency, quality and safety measures. The maximum recommended daily dosage of eluxadoline (Viberzi) in adults is 100 mg for some patients, 75 mg twice daily, taken with food [11]. International Council for Harmonization (ICH) tripartite guidelines suggest impurities in new drug substances identification threshold is 0.1% and the reporting threshold is 0.05% maximum for a daily dose of ≤ 2 g/day [12]. The efficacy and safety of the drug compound primarily depend on the presence of impurities in the drug. Thus, the identification and isolation of these impurities formed during the synthesis of active pharmaceutical ingredient (API) products is crucial to address the key requirements for approval from regulatory agencies.

Due to limited literature availability, the synthesis of these impurities becomes a challenge for manufacturers. Hence, the requirement for the identification of unknown impurities in pharmaceutical substances is inevitable [12-14]. Impurity form-

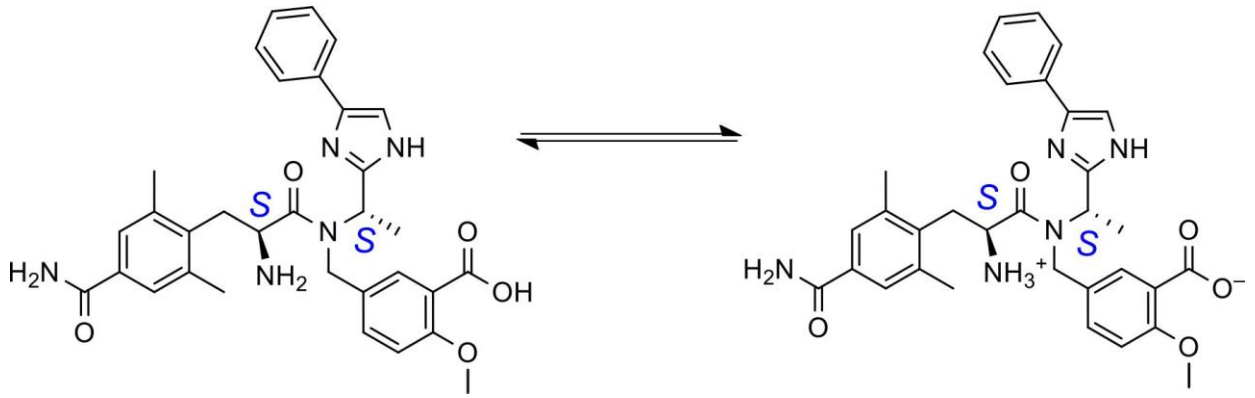
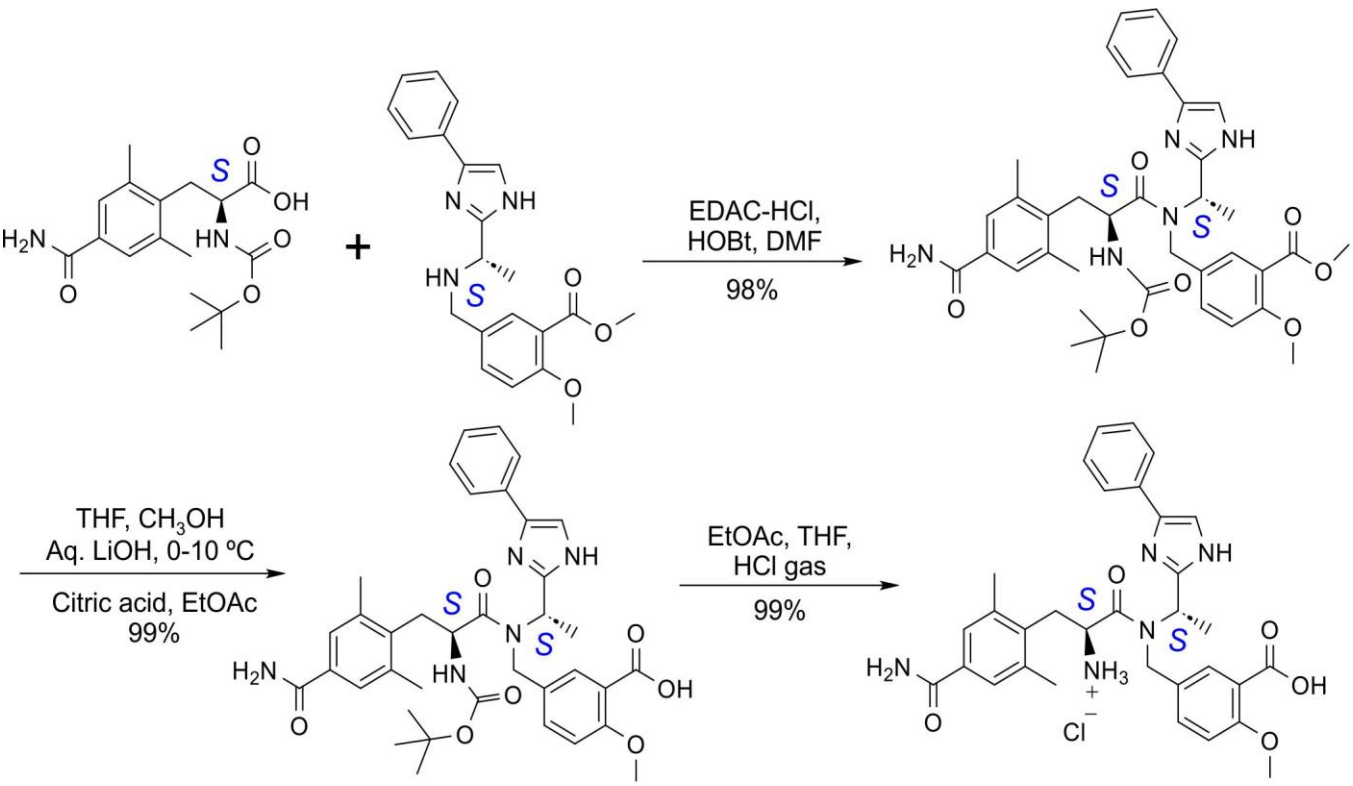


Fig. 1. Chemical structure of eluxadoline

ation and its structural identification become significant tasks in the synthesis of any drug substance, which widely depend on the synthetic route and reaction conditions. Few researchers [15,16] reported the synthesis of eluxadoline, depicted in **Scheme-I**. Later, several researchers synthesized eluxadoline using a similar procedure with slight modifications [17]. Various related impurities observed during the synthesis of eluxadoline have also been reported in the literature [18-20]. During pilot-scale synthesis, unknown impurities were observed in the final product at levels of 0.05 to 0.15% using HPLC. These compounds (Fig. 2) were identified using liquid chromatography-mass spectrometry (LC-MS). This article reports the synthesis and structural illustration of three stereoisomeric impurities identified during the synthesis of eluxadoline [21-23].

EXPERIMENTAL

All the reagents and solvents were purchased from the reputed commercial vendors and used without any further purification. ^{1}H NMR and ^{13}C NMR were recorded in DMSO-d_6, CD_3OD or $CDCl_3$ using a Bruker Ultrashield NMR spectrometer (Bruker CO., Switzerland) at 300 MHz and 75 MHz, respectively. Thin-layer chromatography was carried out on silica gel F-254 plates purchased from Merck with visualization of components by UV light (254 nm). The IR spectra were recorded with an FT-IR spectrophotometer (Perkin-Elmer FTIR-4200). A Shimadzu LC-MS-2020 was used to generate mass spectra (MS). SOR used an Autopol V instrument, analyzing a 1% solution in MeOH at 589 nm and 25 ºC.



Scheme-I: Chemical synthetic route of eluxadoline·HCl [15,16]

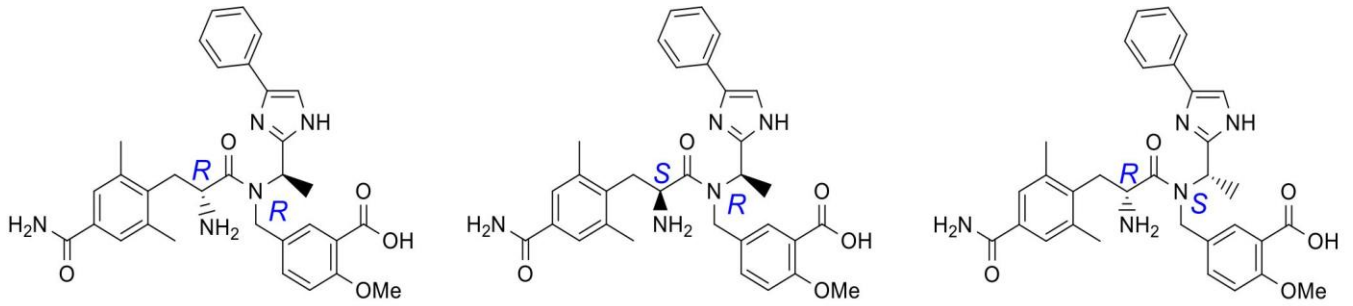


Fig. 2. Structures of new stereoisomeric impurities identified during the process development of eluxadoline

HPLC: The identification of eluxadoline impurities was performed using HPLC chromatography. Details are Waters e2695 HPLC. Column: Cosmosil MS-II C18 (250 × 4.6) mm, 5 µM. Flow rate: 0.8 mL/min l: 210 nm. Injection vol.: 15 µL. Mobile phase A: Water, methanol and orthophosphoric acid in the ratio of 900:100:1 (v/v), disodium phosphate buffer solution (dissolved 0.7 g disodium phosphate in 100 mL water, adjusted to pH 6.8 ± 0.05 with perchloric acid. Mobile phase B: Methanol. Run time: 50 min. Temperature: 50 ºC. Gradient (A: B, 0-15 min: 95:5; 15-34 min: 75:25; 34-42 min: 50:50; 42-42.1 min: 95:5; 42.1-50 min: 95:5).

Chiral HPLC: The other stereoisomer impurities of eluxadoline using chiral HPLC were identified. The HPLC conditions were as follows: Agilent HPLC with Chiralpak IC column (4.6 mm × 250 mm, 5 mm), flow rate 1.0 mL/min, wavelength 210 nm, injection volume 10 µL, mobile phases A (diethyl ether) and B (ethanol) at a 25:75 ratio, with a run time of 40 min at 30 ºC. Eluxadoline retention time was approximately 29.22 min.

Synthesis of compound A ((*R*,*R*)-Eluxadoline enantiomer impurity)

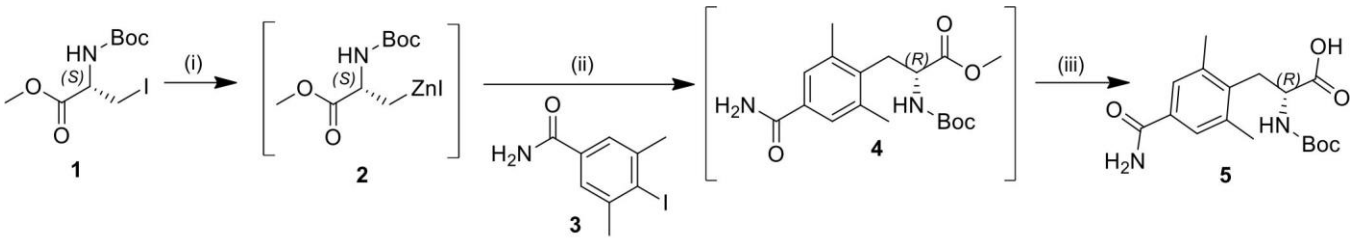
Step-1: Synthesis of 2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)-propionic acid (5)

Synthesis of Zn-iodide complex (2): The Negishi coupling reaction was performed strictly using oven-dried glassware and under a nitrogen atmosphere. Iodine (0.23 g, 0.9 mmol) was added to a mixture containing Zn (2.67 g 40.9 mmol), dry dimethylacetamide (5.5 mL) and dry 2-methyl-THF (3 mL) and then the mixture was cooled to -15 ºC under liquid N_2. Compound **1** (5.98 g, 18.2 mmol) was dissolved in dry *N*,*N'*-dimethylacetamide (DMA, 4.5 mL) and dry 2-methyl-THF (9 mL) was added slowly over 20 min while maintaining the temperature between -15 to -10 ºC. The reaction mixture was warmed slowly to 15 ºC for 1 h and then stirred for 2 h at 15 ºC.

Synthesis of 2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionic acid methyl ester (4): In a separate reaction, compound **3** (2.5 g, 9.1 mmol, dissolved in 5.0 mL of DMA) under N_2 atmosphere was added using syringe to a mixture of dry DMA (3.6 mL), dry 2-methyl-THF (9 mL), tri(*o*-tolyl)phosphine (0.22 g, 0.7 mmol) and *tris*-(dibenzylideneacetone)dipalladium(0) (0.33 g, 0.4 mmol) and the solution was warmed to 45 ºC over 30 min. Then, the above pre-cooled (at 15 ºC), zinc-iodide complex (**2**) was transferred through a cannula slowly to the hot reaction solution at 45 ºC over 30 min. The reaction mixture was stirred at 45-50 ºC and maintained for 3.0 h. After the reaction's completion was confirmed by TLC (solvent system: 50:50 EtOAc/hexane), the mixture was cooled to 25 ºC over 15 min and silica gel (6.25 g) was added with constant stirring for 10 min. The mixture was filtered through a celite bed, washed with EtOAc (30 mL) and the filtrate was quenched with 1 N HCl (20 mL). After the separation of the layers, the aqueous layer was further extracted with EtOAc (20 mL) and the combined organic layers were washed with water (20 mL) and brine (20 mL) and the layers were separated. The organic layer was concentrated to an oily residue under reduced pressure and dissolved in EtOAc (25 mL) and heptane (25 mL). Mixture with constant stirring at RT for 10 min, it was cooled to 5 ºC over 20 min and stirred for 1 h. The precipitated solid was filtered, washed with heptane (10 mL) and dried in a vacuum oven at 40 ºC for 18 h to give compound **4** as a white solid (Yield: 2.86 g, 89%).

Synthesis of 2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionic acid (5): Aqueous LiOH solution (2.5 M, 7.1 mL, 17.9 mmol) was added to a mixture of compound **4** (1.95 g, 3.0 mmol) in MeOH (9 mL, 4.5 vol) over 10 min while maintaining the temperature between 0-10 ºC under N_2. The reaction mixture was heated to 40 ºC over 30 min and maintained for 3 h. After the reaction completion confirmation through HPLC, the reaction mixture was concentrated to 10 mL under reduced pressure. The pH was adjusted to 2 with 20% citric acid solution (~20 mL) while maintaining the temperature between 5-10 ºC. The resulting solution was stirred for 1 h, precipitated solid was filtered, washed with water (10 mL) and dried in a vacuum oven at 40 ºC for 18 h to give compound **5** as a white solid (**Scheme-II**). Yield: 1.50 g, 80%; ^{1}H NMR (300 Hz, DMSO-d_6, δ ppm): 12,59 (s, 1H), 7.80 s, 1H), 7.49 (s, 1H), 7.17-7.22 (t, 2H), 4.05-4.12 (q, 1H), 3.07-3.14 (dd, 1H), 2.92-2.99 (dd, 1H), 2.32 (s, 6H), 1.31 (s, 9H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 173.98, 168.47, 155.73, 138.88, 137.24 (2C), 132.27, 127.52 (2C), 78.54, 53.66, 31.80, 28.60 (2C) & 20.41 (3C); LCMS (ES$^+$): calculated for $C_{17}H_{24}N_2O_5Na^+$:359.15 [M+Na]$^+$; found: 359.33; HPLC purity: 99.75%, Chiral purity by HPLC: 100%.

Step-2: Synthesis of (*R*)-2-methoxy-5-{[1-(4-phenyl-1*H*-imidazol-2-yl)-ethylamino]-methyl}benzoic acid methyl ester (8): A mixture of aldehyde compound **7** (52.0 g, 277



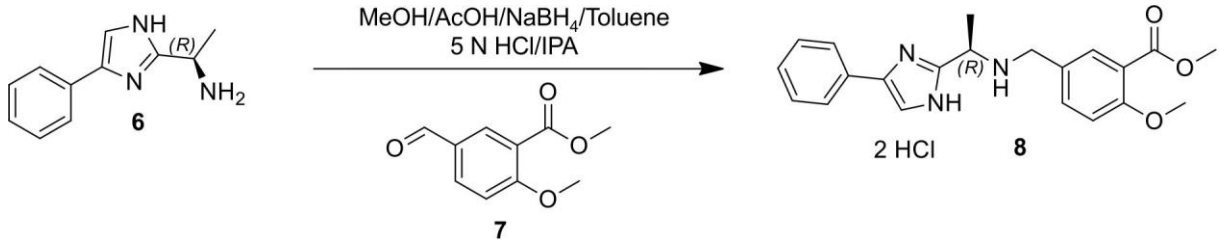
Conditions: (i) Zn/I_2/DMA/2-Me-THF; (ii) P(*o*-tol)$_3$, $Pd_2(dba)_3$/DMA/2-Me-THF; (iii) MeOH/Aq. LiOH

Scheme-II: Synthesis of intermediates during the synthesis of *R,R*-eluxadoline enantiomer

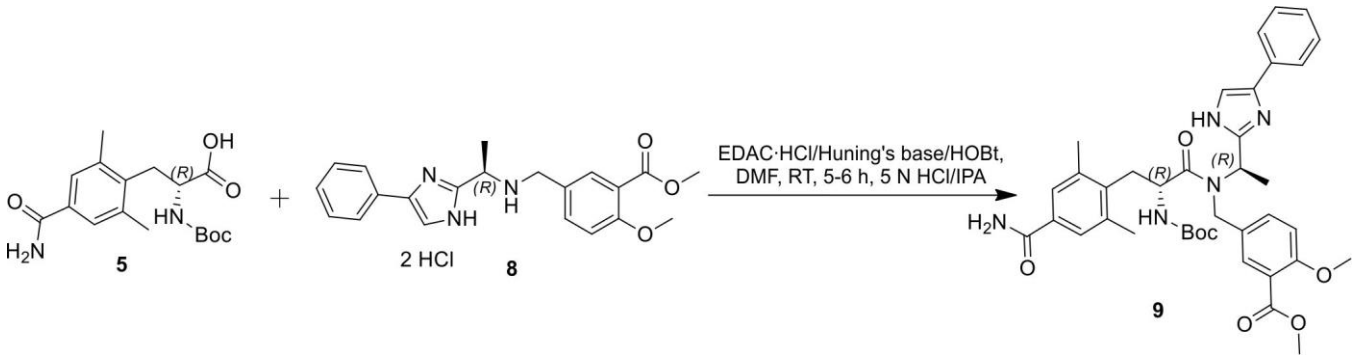
mmol) and amine compound **6** (CAS# 335246-81-6, 50.0 g, 277 mmol) in MeOH (500 mL, 10 vol) was agitated for 30 min at ambient temperature. Acetic acid (17.5 mL, 0.35 vol, 305 mmol) was added to the mixture over 10 min and the resulting mixture was cooled to 0 ºC where a light suspension of $NaBH_4$ (7.5 g, 208 mmol) in toluene (250 mL) was added over 30 min while maintaining the batch temperature below 15 ºC. After confirming the reaction's completion by TLC/HPLC, the reaction mixture's pH was adjusted to pH 6.7 using 1 N HCl in water (150 mL). MeOH was removed by vacuum distillation at 40 ºC. Toluene (150 mL) and water (150 mL) were added. The mixture was agitated for 30 min, settled and the layers were separated. The aqueous layer was re-extracted with toluene twice (75 mL × 2). The combined organic layer was washed with 5% brine (250 mL) and residual water was removed *via* vacuum distillation. HCl IPA (5 N, 100 mL) was added to the above anhydrous toluene solution of the product (300 mL). The resulting solution was slowly added to methyl *tert.*-butyl ether (MTBE, 900 mL). The solid was filtered under nitrogen as it was very hygroscopic. The cake was washed with MTBE (100 mL) and dried in a vacuum oven at ambient temperature with a small nitrogen bleed for 18 h to obtain compound **8** as a white solid (**Scheme-III**). Yield: 103.6 g; 88.5%. ^{1}H NMR (300 Hz, MeOH d-4, δ ppm): 7.69-7.71 (d, 3H), 7.44-7.47 (d, 1H), 7.36-7.41 (t, 2H), 7.32 (s, 1H), 7.22-7.27 (t, 1H), 7.01-7.04 (d, 1H), 3.97-4.04 (q,1H), 3.83 (s, 6H), 3.64 (s, 2H), 1.48-1.50 (d, 3H); ^{13}C 75.49 nm, (75 Hz, MeOD d_4, δ ppm): 1167.00, 158.23, 151.97, 137.55, 133.71, 132.97, 131.35, 131.18, 128.45 (2C), 126.48, 124.46 (2C), 119.32, 115.76, 112.01, 55.13, 51.42, 51.18, 50.27 & 20.35; LCMS (ES^+): calculated for $C_{21}H_{23}N_3O_3^+$: 366,18 $[M+H]^+$; found: 366.25; HPLC purity: 95.68%, Chiral purity by HPLC:99.87%.

Step-3: Synthesis of 5-({[2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl][1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid methyl ester hydrochloride (9): DMF (14 mL, 4.8 vol) was slowly added to a mixture of compound **8** (free base, 3.0 g, 8.2 mmol), compound **5** (3.01 g, 9.0 mmol) and hydroxybenzotriazole (HOBt) (85% assay, 1.39 g, 8.2 mmol) while maintaining the temperature between 8-15 ºC under N_2 atmosphere. The reaction mixture was stirred for 30 min and then a solution of EDAC·HCl (4.25 g, 22.2 mmol) dissolved in DMF (4.8 mL) and N, N'-diisopropylethylamine (5.00 mL, 28.7 mmol) was added over 45 min while keeping the temperature below 20 ºC. The reaction mixture was warmed to room temperature for 1 h and then stirred for 3 h. After the complete reaction was confirmed by TLC, IPAc (15 mL) and water (18 mL) were added sequentially throughout 30 min while keeping the temperature below 25 ºC. The layers were separated and the bottom aqueous layer was extracted three times with IPAc (3 × 15 mL) and separated. The combined rich IPAc layer was washed with 5% $NaHCO_3$ (2 × 25 mL), water (25 mL) and 5% brine (25 mL). The organic layer was separated and concentrated to ~6.0 mL under vacuum (residual water). The rich IPAc layer was diluted with fresh IPAc (30 mL) and MeOH (4.5 mL) and kept stirring. IPA·HCl (5 N, 1.8 mL, 9.4 mmol) was added over 30 min while maintaining the temperature below 20 ºC and the mixture was stirred at room temperature for 1 h. The solid precipitate was filtered, washed with IPAc (6.0 mL) and dried in a vacuum oven at 40 ºC for 12 h to give compound **9** as a white solid (**Scheme-IV**). Yield: 5.40 g, 91%; ^{1}H NMR (300 Hz, MeOH-d_4, δ ppm): 7.51-7.59 (m, 3H), 7.34-7.46 (m, 8H), 7.18 (s, 2H), 6.53-6.56 (d, 1H), 5.02-5.09 (q, 1H), 4.85-4.90 (m, 1H), 3.67 (s, 2H), 3.59 (s, 3H), 3.34 (s, 3H), 3.01-3.07 (dd, 2H), 2.33 (s, 6H), 1.48 (s, 9H) & 1.16 (s, 3H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 174.01, 170.49, 166.24, 158.09, 157.60, 145.93, 138.22 (2C), 137.80, 136.83, 136.50, 134.31, 133.91, 132.57, 130.98, 128.92, 127.69, 127.28, 125.81, 125.56 (2C), 119.31, 114.28, 111.97, 80.87, 60.14, 54.71, 51.08, 49.23, 42.99, 35.88, 27.37 (3C), 18.81 (2C) & 12.56; LCMS (ES^+): calculated for $C_{382}H_{46}N_5O_7Cl^+$: 684.33 $[M+H]^+$; found: 684.3; HPLC purity: 99.73%.

Step-4: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl][1-(4-phenyl-1*H*-imidazol-2-yl)-ethyl]amino}methyl)-2-methoxybenzoic acid methyl ester dihydrochloride (10): In the reaction mixture of compound



Scheme-III: Synthesis of intermediate **8** during the synthesis of *R,R*-eluxadoline enantiomer



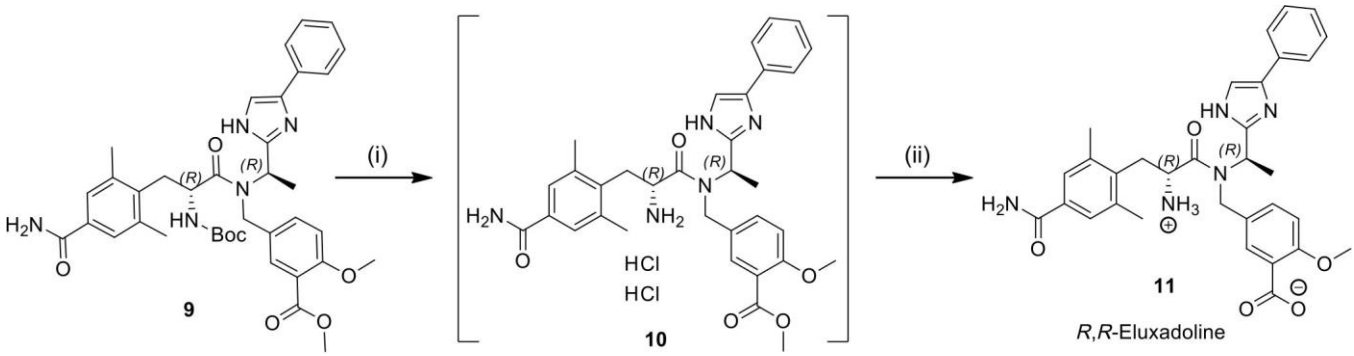
Scheme-IV: Synthesis of intermediate **9** during the synthesis of *R,R*-eluxadoline enantiomer

9 (5.00 g, 6.9 mmol) and MeOH (35 mL, 7 vol) HCl gas was bubbled slowly over 30 min while maintaining the temperature below 15 ºC. The clear reaction mixture was warmed to room temperature for 1 h and then stirred at 18-23 ºC for 2 h. After complete reaction confirmation using TLC, the reaction mixture was concentrated to 15-20 mL under vacuum (at 20-25 ºC) and cooled to 10 ºC for 30 min. MTBE (50 mL) was added over 30 min and the mixture was stirred at 10 ºC for 1 h. The precipitated solid was filtered, washed with MTBE (15 mL) and dried in a vacuum oven at 40 ºC for 12 h to give compound **10** as a white solid (yield: 4.0 g, 87%).

Step-5: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl][1-(4-phenyl-1*H*-imidazol-2-yl)-ethyl]amino}methyl)-2-methoxybenzoic acid (11): Aqueous LiOH solution (2.5 M, 11 mL, 27.5 mmol) was added slowly over 20 min to a mixture of compound **10** (3.00 g, 4.6 mmol) and MeOH (14 mL, 4.5 vol) while maintaining the temperature between 0-10 ºC under N_2 atmosphere (pH of the mixture should be between 11-13). The reaction mixture was heated to 40 ºC over 30 min and maintained for 3 h. After confirming the reaction's completion using TLC, MeOH was concentrated under vacuum and the resulting rich aqueous solution (pH ~12) was added slowly to NaH_2PO_4 monobasic buffer solution (1 N, 15 mL; pH ~4.5) while maintaining the temperature between 5-10 ºC (pH of the mixture will be ~5.5). The pH of the mixture was further adjusted to 6.8 with 2 N NaOH solution (20 mL). The solid precipitate was filtered, washed with water (15 mL) and dried in a vacuum oven at 40 ºC for 12 h to give compound **11** as **A** a white solid compound (**Scheme-V**). Yield: 1.85 g; 70%; ^{1}H NMR (300 Hz, DMSO-d_6, δ ppm): 6.762-7.683 (m, 12H), 5.155-5.172 (m, 1H), 4.494-4.619 (m, 1H), 3.679 & 3.692 (2s, 1H), 3.510-3.570 (t, 1H), 2.875-3.169 (m, 1H), 2.080 & 2.358 (2s, 2H), 1.009-1.433 (2d, 1H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 175.14, 167.97, 156.24, 155.80, 146.84, 138.53, 137.81, 137.10, 136.87, 132.04, 131.63, 130.07, 128.94,128.40, 127.32, 126.98, 126.05, 124.28, 111.47, 59.74, 55.55, 55.59, 50.31, 50.96, 44.70, 34.39, 19.96, 20.19, 16.96, 17.40; HRMS (ES$^+$): calculated for $C_{32}H_{35}N_5O_5^+$: 570.2710 $[M+H]^+$; found: 570.2720; HPLC purity: 92.13%, Chiral purity by HPLC: 99.31%.

Synthesis of compound B [(*S,R*)-eluxadoline diastereomer]

Step-1: Synthesis of 5-({[2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl][1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid methyl ester hydrochloride (13): DMF (23 mL, 4.8 vol) was added slowly to a mixture of acid **12** (4.73 g, 14.1 mmol), previously synthesized compound **8** (free base, 4.71 g, 12.9 mmol), HOBt (85% assay, 2.37 g, 12.9 mmol) while maintaining the temperature between 8-15 ºC under N_2. The reaction mixture was stirred for 30 min and then a solution of EDAC·HCl (7.27 g, 34.8 mmol) dissolved in DMF (7.5 mL) and *N,N'*-diisopropylethylamine (8.57 mL, 45.1 mmol) was added over 45 min while keeping the temperature below 20 ºC. The reaction mixture was warmed gradually to room temperature for 1 h and then stirred for 3 h. After confirmation of complete reaction by TLC, IPAc (25 mL) and water (28 mL) were added sequentially throughout 30 min while keeping the temperature below 25 ºC. The layers were separated and the bottom aqueous layer was extracted with IPAc (3 × 20 mL)



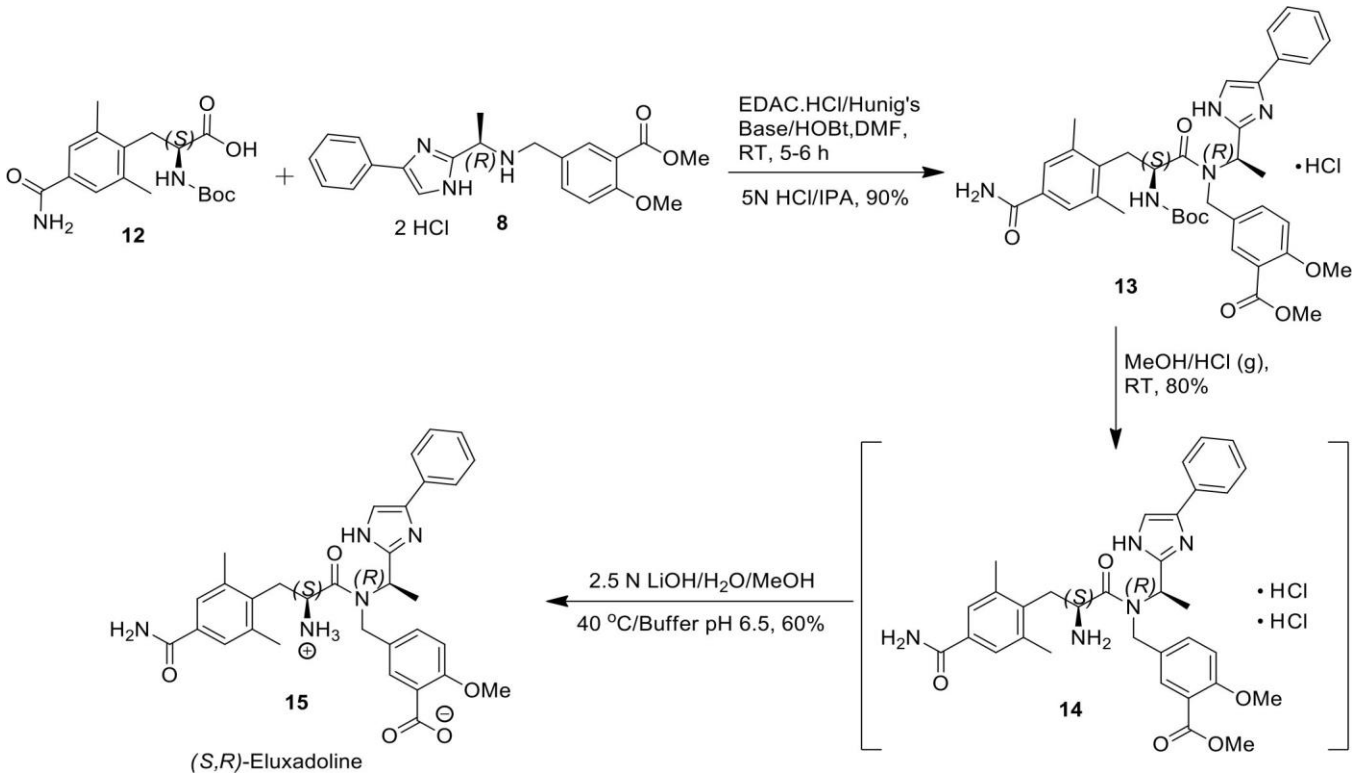
Conditions: (i) MeOH/HCl (g); (ii) 2.5 N LiOH/H_2O/MeOH, 40 °C/Buffer pH 6.5

Scheme-V: Synthesis of *R,R*-eluxadoline impurity (compound **A**) (Steps 4 & 5)

and separated. The combined rich IPAc layer was washed with 5% $NaHCO_3$ (2 × 30 mL), water (30 mL) and 5% brine (30 mL). The organic layer was separated and concentrated to ~15 mL under vacuum. IPAc (50 mL) and MeOH (7 mL) were added to the rich concentrate with constant stirring. HCl in IPA (5 N, 2.8 mL, 14.8 mmol) was added over 0.5 h and the mixture was stirred at room temperature for 1.0 h. The precipitated solid was filtered, washed with IPAc (9.5 mL) and dried in a vacuum oven at 40 ºC for 12 h to give compound **13** as a white solid. Yield: 8.33 g; 90%; ^{1}H NMR (300 Hz, MeOH-d_4, δ ppm): 6.79-7.46 (m, 11H), 5.57-5.64 (q, 1H), 4.80-4.86 (t, 1H), 3.86-4.36 (m, 2H), 3.63 (s, 3H), 3.58 (s, 3H), 2.99-3.20 (dd, 2H), 2.25 (s, 6H), 1.47-1.50 (s, 3H) & 1.13-1.23 (m, 9H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 173.29, 171.15, 170.97, 166.49, 158.49, 155.63, 145.86, 137.76 (2C), 137.43, 133.78, 132.33, 131.94, 129.39, 128.98 (2C), 127.26 (2C), 126.89, 126.29, 125.72 (2C), 119.90, 112.38, 79.44, 67.68, 55.08, 51.21, 49.76, 46.68, 32.65, 27.25 (3C), 20.66 (2C) & 19.39; LCMS (ES^+): calculated for $C_{382}H_{46}N_5O_7Cl^+$: 684.33 $[M+H]^+$; found: 684.3; HPLC purity: 98.52%.

Step-2: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl]-[1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxy-benzoic acid methyl ester dihydrochloride (14): In a mixture of compound **13** (6.83 g, 9.5 mmol) and MeOH (50 mL, 7 vol), HCl gas was bubbled slowly over 20 min while maintaining the temperature below 20 ºC. The reaction mixture was allowed to gradually warm to room temperature for 1 h and then stirred for 2 h (bath temp. 18-23 ºC). After confirmation of the reaction's completion by HPLC, the reaction mixture was concentrated to 25 mL (bath temperature 20-25 ºC) under vacuum and cooled to 10 ºC over 30 min. MTBE (60 mL) was added for 30 min and the mixture was stirred for 1 h at 10 ºC. The solid precipitate was filtered, washed with MTBE (20 mL) and dried in a vacuum oven at 40 ºC for 18 h to give compound **14** as a white solid (yield: 5.0 g, 80%).

Step-3: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl]-[1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid (15): Aqueous LiOH solution (2.5 M, 15 mL, 36.6 mmol) was added slowly over 20 min to a mixture of compound **14** (4.00 g, 6.1 mmol) and MeOH (18 mL, 4.5 vol) while maintaining the temperature between 0-10 ºC under N_2 atmosphere. The reaction mixture was heated to 35-40 ºC over 30 min and maintained for 3-4 h. After confirmation of reaction completion by HPLC, MeOH was concentrated under vacuum and the resulting rich aqueous solution was added slowly over 30 min to NaH_2PO_4 monobasic buffer solution (1 N, 40 mL; pH~4.5) while maintaining the temperature between 5-10 ºC (pH of the mixture ~5.5). The pH of the mixture was further adjusted to 6.8 with 2 N NaOH solution (~20 mL). The precipitated solid was filtered, washed with water (20 mL) and dried in a vacuum oven at 40 ºC for 18 h to give compound **15** as compound B white solid with 94.49% purity by HPLC (**Scheme-VI**). Yield: 2.10 g, 60%; ^{1}H NMR (300 Hz, DMSO-d_6, δ ppm): 6.758-7.857 (m, 12H), 5.858-5.910 (m, 1H), 4.172-4.457 (m, 1H), 3.667 & 3.722 (2s, 1H), 3.545-3.509 (t, 1H), 2.841-3.024 (m, 1H), 2.197 & 2.353 (2s, 2H), 1.299-1.470 (d, 1H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 175.07, 168.04, 167.94, 167.63, 156.43, 146.47, 138.19, 137.14, 136.83, 131.87, 130.81, 129.50, 128.02, 128.30, 127.51, 127.11, 125.93, 124.25, 122.54, 55.58, 50.64, 46.81, 44.59, 34.41, 35.23, 19.94, 20.50, 16.68 & 18.34; HRMS (ES^+): calculated for $C_{32}H_{35}N_5O_5^+$: 570.2710 $[M+H]^+$; found: 570.2720; HPLC purity: 94.49%; Chiral purity by HPLC: 100%.



Scheme-VI: Synthesis of diastereomer *S*,*R*-eluxadoline impurity (compound **B**)

Synthesis of compound C ((*R,S*)-eluxadoline diastereomer)

Step-1: Synthesis of 5-({[2-*tert*-butoxycarbonylamino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl][1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid methyl ester hydrochloride (17): DMF (14 mL, 4.8 vol) was added slowly to a mixture of previously synthesized compound **5** (3.01 g, 9.0 mmol), Amine compound **16** (3.00 g, 8.2 mmol) and HOBt (85% assay, 1.39 g, 8.2 mmol) while maintaining the temperature between 8-15 ºC under N_2 atmosphere. The reaction mixture was stirred for 30 min and then a solution of EDAC·HCl (4.25 g, 22.2 mmol) dissolved in DMF (4.8 mL) and *N,N'*-diisopropylethylamine (5.0 mL, 28.7 mmol) was added over 45 min while keeping the temperature below 20 ºC. The reaction mixture was warmed gradually to room temperature for 1 h and then stirred for 3 h. After confirming the reaction's completion by TLC, IPAc (15 mL) and water (18 mL) were added sequentially for 30 min, while keeping the temperature below 25 ºC. The layers were separated and the bottom aqueous layer was extracted with IPAc (3 × 15 mL) and separated. The combined rich IPAc layer was washed with 5% $NaHCO_3$ (2 × 20 mL), water (20 mL) and 5% brine (20 mL). The organic layer was separated and concentrated to ~6.0 mL under vacuum. The rich IPAc layer was diluted with IPAc (30 mL) and MeOH (4.5 mL) and stirred. HCl in IPA (5 N, 1.8 mL, 9.4 mmol) was added over 30 min and the mixture was stirred for 1.0 h. The precipitated solid was filtered, washed with IPAc (6.0 mL) and dried in a vacuum oven at 40 ºC for 12 h, resulting in compound **17** as a white solid (yield: 5.53 g; 96%); ^{1}H NMR (300 Hz, MeOH-d_4, δ ppm): 7.45-7.49 (m, 3H), 6.80-7.40 (m, 8H), 5.57-5.64 (q1H), 4.75-4.85 (t, 1H), 3.89-4.37 (m, 2H), 3.63 (s, 3H), 3.58 (s, 3H), 3.03-3.17 (m, 2H), 2.24 (s, 6H), 1.48-1.50 (d, 3H) & 0.90-1.22 (s, 9H); ^{13}C NMR (75 Hz, DMSO-d_6, δ ppm): 173.29, 171.59, 170.99, 166.50, 158.49, 155.63, 145.86, 137.76 (2C), 137.43, 133.80, 132.32, 131.95, 129.37, 128.97 (2C), 127.26 (2C), 126.90, 126.33, 125.71 (2C), 119.89, 114.78, 112.38, 79.45, 60.16, 51.21, 49.76, 46.68, 32.65, 27.25 (3C), 26.71, 19.11 (2C) & 13.12; LCMS (ES$^+$): calculated for $C_{38}H_{46}N_5O_7Cl^+$: 684.33 $[M+H]^+$; found: 684.3; HPLC purity: 93.98%, chiral purity by HPLC: 99.87% .

Step-2: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl]-[1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid methyl ester dihydrochloride (18): In a mixture of compound **17** (4.00 g, 5.5 mmol) and MeOH (28 mL, 7 vol), HCl gas was bubbled slowly over 20 min while maintaining the temperature below 20 ºC. The reaction mixture was warmed to room temperature gradually for 1 h and then stirred at 18-23 ºC for 2 h. After confirming the reaction's completion by TLC, the reaction mixture was concentrated to 25 mL under vacuum (bath temp., 20-25 ºC) and cooled to 10 ºC over 30 min. MTBE (30 mL) was added over 30 min and the mixture was stirred for 1 h at 10 ºC. The precipitated solid was filtered, washed with MTBE (10 mL) and dried in a vacuum oven at 40 ºC for 18 h, resulting in compound **18** as a white solid (yield: 2.92 g; 80%).

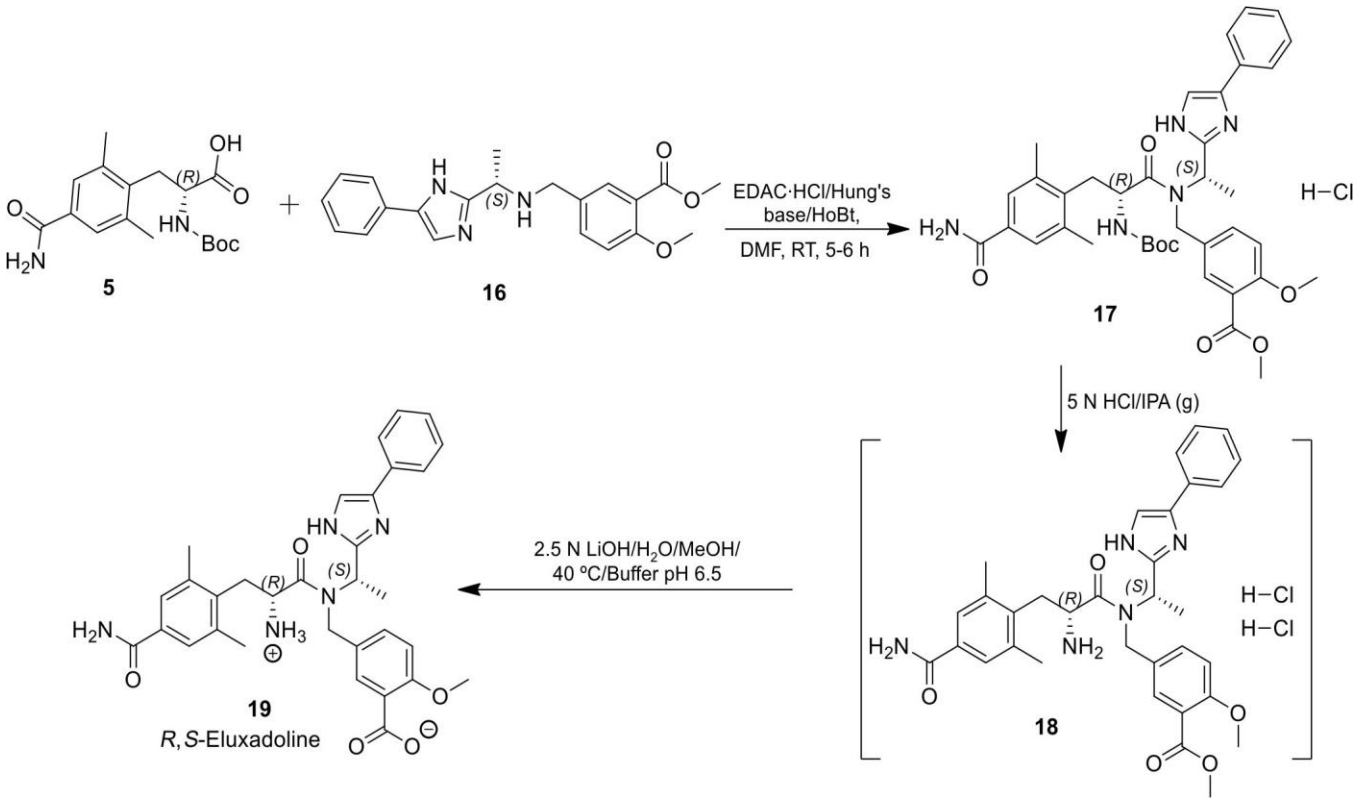
Step-3: Synthesis of 5-({[2-amino-3-(4-carbamoyl-2,6-dimethyl-phenyl)propionyl]-[1-(4-phenyl-1*H*-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid (C): Aqueous LiOH solution (2.5M, 7.3 mL, 18.3 mmol) was added slowly over 20 min to a mixture of compound **18** (2.0 g, 3.0 mmol) and MeOH (10 mL, 4.5 vol) while maintaining the temperature between 0-10 ºC under N_2 atmosphere. The reaction mixture was heated to 40 ºC over 30 min and maintained for 3 h. After confirmation of complete reaction by TLC, MeOH was concentrated under vacuum, resulting rich aqueous solution that was added slowly to NaH_2PO_4 monobasic buffer solution (1 N, 30 mL; pH ~4.5) while maintaining the temperature between 5-10 ºC (pH of the mixture ~5.5). The pH of the mixture was further adjusted to 6.8 with 2 N NaOH (~20 mL). The precipitated solid was filtered, washed with water (10 mL) and dried in a vacuum oven at 40 ºC for 18 h to result in compound **19** as compound C a white solid (**Scheme-VII**). Yield: 0.67 g; 40% with 96.78% purity by HPLC; ^{1}H NMR (300 Hz, DMSO-d_6, δ ppm): 6.758-7.860 (m, 12H), 5.858-5.910 (m, 1H), 4.170-4.461 (m, 1H), 3.721 & 3.666 (2s, 1H), 3.505-3.541 (t, 1H), 2.839-3.020 (m, 1H), 2.196 & 2.353 (2s, 2H), 1.299-1.467 (d, 1H); ^{13}C NMR (75 Hz, CD_3OD, δ ppm): 175.29, 175.15,168.06, 167.96, 167.57, 156.42, 146.50, 138.25, 137.15, 136.83, 131.86, 129.53, 128.98, 128.32, 127.51, 127.11, 125.94, 124.32, 124.26, 112.18, 55.59, 55.66, 50.68, 46.81, 44.61, 35.280, 19.95, 20.52, 16.70, 18.34; HRMS (ES$^+$): calculated for $C_{32}H_{35}N_5O_5^+$: 570.2710 $[M+H]^+$; found: 570.2717; HPLC purity: 96.78%, chiral purity by HPLC: 100%.

RESULTS AND DISCUSSION

During pilot-scale synthesis, unknown impurities were observed in the final eluxadoline (API) at levels of 0.05 to 0.15% using HPLC. These impurities were identified using liquid chromatography-mass spectrometry (LC-MS) and named as compounds **A**-**C**. Upon identifying, the objective is to synthesize and characterize each impurity in a pure form.

Eluxadoline (*R,R*)-enantiomer impurity (A): This impurity is formed due to the carryover of traces of *R*-isomers present in intermediates **12** and **16** or epimerization at the α-carbon of the amino acid moiety under acidic or basic conditions. Synthesis of this impurity involves multiple steps for the preparation of intermediates **5** and **8** as shown in **Schemes II** and **III**, respectively. Compound **2** was synthesized *via* a Negishi coupling reaction conducted under a N_2 atmosphere and subsequently converted to compound **5** through a hydrolysis (**Scheme-II**, step-1). The reductive amination of comercially available aldehyde **7** and amine **6** afforded intermediate **8**. Later, the coupling of compounds **5** and **8**, followed by Boc deprotection using MeOH/HCl and hydrolysis using lithium hydroxide, resulted in eluxadoline (*R,R*) enantiomer impurity **A** as shown in **Scheme-V** (steps 4 & 5). The formation of enantiomer impurity compound **A** was confirmed by MS, showing a molecular ion peak at 570.10 [M+1] in positive ionization, indicating a molecular weight of 569.65 g/mol. The formation of the product was confirmed by ^{1}H NMR, ^{13}C NMR and IR spectroscopic data. The observed specific optical rotation (SOR) of (*R,R*)-eluxadoline is -66.56, whereas that of (*S,S*)-eluxadoline is +66.38 measured under identical conditions.

Eluxadoline (*S,R*)-diastereomer impurity (B): This impurity is the (*S,R*) diastereomer of eluxadoline and is designated



Scheme-VII: Synthesis of diastereomer *R*,*S*-eluxadoline impurity (compound **C**)

as compound **B**. The synthetic protocol involves three steps. The amide coupling of commercially available 4-(aminocarbonyl)-N-[(1,1-dimethylethoxy)carbonyl]-2,6-dimethyl-L-phenylalanine (**12**) and intermediate **8** using EDC·HCl as a coupling agent afforded **13** as shown in **Scheme-VI**. Subsequent Boc deprotection, followed by ester hydrolysis, afforded eluxadoline (*S*,*R*)- diastereomer impurity (**B**). The product was characterized by MS, revealing a molecular ion peak of 570.0 [M+1] and 568.3 [M-1], indicating a molecular weight of 569.65 g/mol. FTIR analysis identified key peaks at 3167.33 cm^{-1} (amide N-H *str.*), 2965.61 cm^{-1} (arom. C-H *str.*), 1658.01 cm^{-1} (amide C=O *str.*), 1611.34 cm^{-1} (acid C=O *str.*), 1492.09 cm^{-1} (arom. C=C *str.*), 1286.52 cm^{-1} (alkyl C-N *str.*). The observed specific optical rotation of (*S*,*R*)-eluxadoline is +36.13, whereas that of (*S*,*S*)-eluxadoline is +66.38, measured under identical conditions.

Eluxadoline (*R*,*S*)-diastereomer impurity (C): The synthetic protocol also involves three steps. The amide coupling of commercially available (*S*)-methyl-2-methoxy-5-((1-(4-phenyl-1*H*-imidazol-2-yl)ethylamino)methyl)benzoate (**16**) and acid **5** afforded compound **17**, which was further treated with 5 N HCl solution, followed by lithium hydroxide, resulting in eluxadoline (*R*,*S*) diastereomer impurity (**C**) as depicted in **Scheme-VII**. Synthesis of compound **C** was confirmed by MS, showing a molecular ion peak at 570.1 [M+1] in positive mode and 598.3 [M-1] in negative mode, consistent with a molecular weight of 569.65 g/mol. FTIR analysis identified key peaks at 3171.63 cm^{-1} (amide N-H *str.*), 2922.35 cm^{-1} (arom. C-H *str.*), 1656.51 cm^{-1} (amide C=O *str.*), 1611.02 cm^{-1} (acid C=O *str.*), 1494.52 cm^{-1} (arom. C=C *str.*), 1287.13 cm^{-1} (alkyl C-N *str.*). The observed specific optical rotation of (*R*,*S*)-eluxadoline is -40.95, whereas that of (*S*,*S*)-eluxadoline is +66.38, measured under identical conditions.

All the newly synthesized stereoisomeric impurities were spiked with eluxadoline API using chiral HPLC chromatography. Fig. 3 represents a chromatogram for eluxadoline enantiomer and diastereomer impurity identification. To differentiate between enantiomers and diastereomers, specific optical rotation (SOR) measurements were performed using an Autopol V polarimeter (Rudolph Research Analytical). The measurements were carried out under anhydrous conditions using a 1% (w/v) solution in methanol at a wavelength of 589 nm and at 25 ºC. The corresponding SOR values are summarized in Table-1.

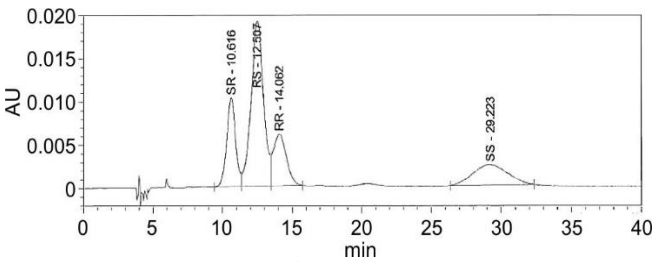


Fig. 3. HPLC chromatogram of eluxadoline stereoisomeric impurities

Conclusion

In summary, the stereoisomers of eluxadoline were successfully identified, synthesized and structurally confirmed for the first time using comprehensive analytical techniques like ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry. Together with previous work [24], this study completes the synthesis and

TABLE-1
DETAILS OF SOR, MELTING POINT BY DSC AND ENANTIOMERIC EXCESS BY HPLC FOR ELUXADOLINE STEREOISOMERIC IMPURITIES

Enantiomer	m.p. (ºC)	Calculated SOR for 100% potency	% of Enantiomeric excess (ee)
S,*S*-Eluxadoline	199.66	66.38	100
R,*R*-Eluxadoline	207.47	-66.56	98.62
S,*R*-Eluxadoline	174.47	36.13	100
R,*S*-Eluxadoline	173.46	-40.95	100

characterization of all eluxadoline stereoisomers, thereby establishing a complete stereoisomeric profile in accordance with ICH regulatory requirements [25,26]. These findings provide access to reference standards for the stereoisomers and offer valuable insights for future pharmaceutical development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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