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Process development of clinical anti-HBV drug Y101: identification and synthesis of novel impurities

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Abstract Nine novel process impurities of *N*-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-L-phenylalaninol (**Y101**) observed during the laboratory optimization and later during its bulk synthesis are described in this article. The impurities were monitored by HPLC, and their structures were tentatively assigned on the basis of fragmentation patterns in LC−MS/MS and NMR spectroscopies. All of the impurities were synthesized, and their assigned constitutions were confirmed by co-injection in HPLC. In addition to the formation, synthesis, and characterization, the strategy for minimizing these impurities to a level accepted by the International Conference on Harmonisation (ICH) was also described.

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Introduction

Y101 (Fig. 1) was developed for the treatment of chronic hepatitis B associated with an increase of alanine aminotransferase (ALT) and evidence of hepatitis B viral replication as well as active liver inflammation, especially for the treatment of chronic hepatitis B with the resistance of nucleoside analogues [1–5]. The clinical trial application of **Y101** was approved by the China Food and Drug Administration (CFDA) at the end of 2013 (No. 2013L02491). Furthermore, a Phase 1 clinical trial of **Y101** is now underway in the First Affiliated Hospital of Soochow University.

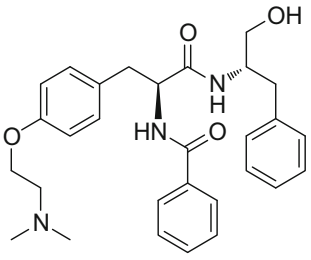
The control of impurities observed in the active pharmaceutical ingredient (API) is critical in delivering an API of high quality. To ensure the desired drug metabolism, safety, and that clinical studies are not jeopardized by inconsistent purity or impurities having potential harmful toxicological properties, the detection, identification, quantification, and control of these impurities originating in the manufacturing process have become an important element of drug development [6].

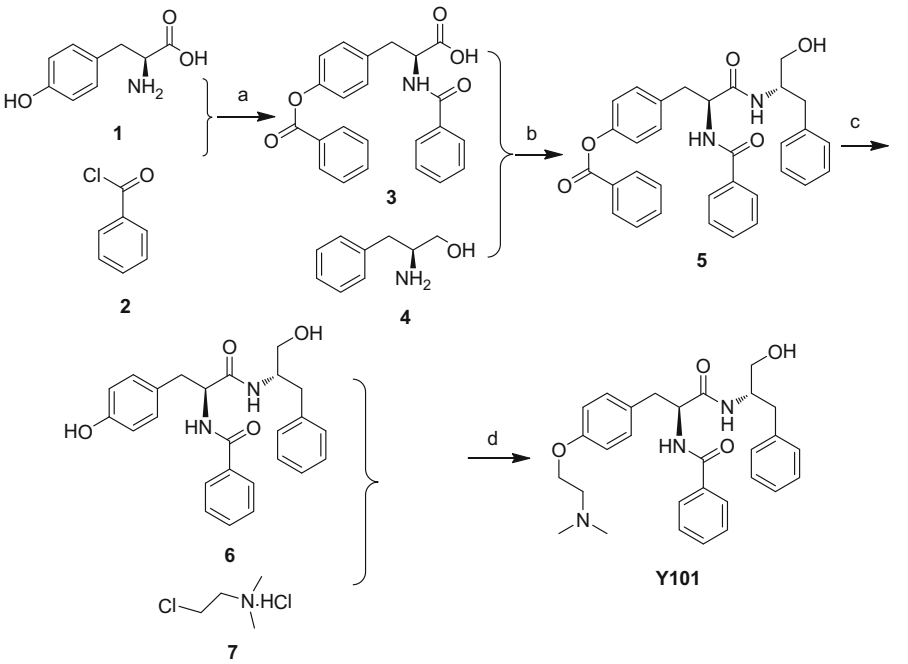
When **Y101** was synthesized by a practical and scalable synthetic method (Scheme 1), nine unknown impurities with area percentages ranging from 0.02 to 0.13 % were detected by high performance liquid chromatography (HPLC) [7]. It is mandatory for the manufacturer to identify and characterize all the unknown impurities that are present in an API even below a level of 0.05 % [6]. In this context, a comprehensive study has been undertaken to identify, synthesize, and characterize all the nine impurities present in the laboratory batches of **Y101** using spectroscopic and spectrometric techniques.

Materials and methods

All materials were purchased from commercial suppliers. Unless specified otherwise, all reagents and solvents were supplied by their manufacturers. Melting points were determined with an X-4 melting point apparatus. The ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and two-dimensional NMR (2D NMR) spectra were measured in deuterated dimethyl sulfoxide (DMSO-d_6) on an INOVA 400 MHz Fourier transform (FT) NMR spectrometer. Chemical shifts are reported

Fig. 1 Structure of **Y101**





Scheme 1 Reaction conditions: (*a*) 0.25-M NaOH, 2 °C, 1 h, 90-% yield; (*b*) IBCF, NMM, DMF, CH_2Cl_2, −10 °C, 1 h, 81-% yield; (*c*) 2.0-M NaOH, DMF, 30 °C, 4 h, 82-% yield; (*d*) K_2CO_3, 1,4-dioxane, 90 °C, 2 h, 90-% yield

in δ (ppm) relative to trimethylsilane (TMS; δ 0.0) for ^{1}H NMR spectra, and DMSO-d_6 (δ 39.50) for ^{13}C NMR spectra. Mass spectra were determined on an Hewlett Packard 1100 LC-MSD mass spectrometer.

Synthesis of compound *N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosine (Impurity G)

*Synthesis of intermediate N-benzoyl-L-tyrosine methyl ester (**9**)*

Isobutyl chloroformate (IBCF) (4.1 g, 30 mmol) was added dropwise to the mixture of benzoic acid (3.05 g, 25 mmol), **8** (6.95 g, 30 mmol), 4-methylmorpholine (NMM) (5.1 g, 50 mmol), and DMF (100 mL) in CH_2Cl_2 (250 mL) at −10 °C within 30 min. The mixture was stirred for 1.5 h, and the bulk of CH_2Cl_2 was removed in vacuo. The residue was dissolved in ethyl acetate and washed sequentially with water, 5-% HCl, saturated $NaHCO_3$ solution and brine, and dried with $MgSO_4$. Filtration and solvent evaporation gave a residue which was recrystallized from ethyl acetate to afford target intermediate **9** (5.98 g, 80 %). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.20 (s, 1H), 8.77 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 7.2 Hz, 2H), 7.53 (t, J = 7.2 Hz, 1H), 7.53 (t, J = 7.2 Hz, 1H), 7.47 (t, J = 7.6 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 6.66 (d, J = 8.4 Hz, 2H), 4.61-4.55 (m, 1H), 3.62 (s, 3H), 3.08–2.95 (m, 2H).

Synthesis of impurity ***G***

Intermediate **9** (4.49 g, 15 mmol) was dissolved in 50 mL of 1,4-dioxane under argon atmosphere. **7** (2.6 g, 18 mmol) and potassium carbonate (12.42 g, 90 mmol) were added. The reaction mixture was stirred at 90 °C for 2 h. The resulting mixture was diluted with water (200 mL), extracted with ethyl acetate (300 mL), then the organic layer was washed with brine (200 mL), dried over $MgSO_4$, filtered and concentrated. The crude material was dissolved in 100 mL of methanol and reacted with 20 mL of 2-M NaOH at 30 °C for 3 h. The resulting mixture was cooled to 0 °C and its pH was adjusted to 6 with 10-% acetic acid. The bulk of methanol was removed in vacuo, the residue was dissolved in n-butyl alcohol and washed with water. The n-butyl alcohol layer was dried over $MgSO_4$, filtered and concentrated, and the crude material was recrystallized from $CHCl_3$-EtOAc to give impurity **G** (3.74 g, 99.22 % purity) in 70-% yield. Melting point (Mp): 154–156 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, $J = 8.0$ Hz, 1H, 1″-CONH), 7.79 (m, 2H, H-3″, 7″), 7.51 (ddd, $J = 6.4$, 3.8, 1.4 Hz, 1H, H-5″), 7.47–7.40 (m, 2H, H-6″, 4″), 7.19 (d, $J = 8.7$ Hz, 2H, H-5, 9), 6.81 (d, $J = 8.7$ Hz, 2H, H-6, 8), 4.49 (ddd, $J = 9.6$, 8.1, 4.3 Hz, 1H, H-2), 4.05 (t, $J = 5.7$ Hz, 2H, OCH_2CH_2N), 3.07 (ddd, $J = 23.4$, 13.7, 7.0 Hz, 2H, H-3a, 3b), 2.82 (t, $J = 5.6$ Hz, 2H, OCH_2CH_2N), 2.35 (s, 6H, $N(CH_3)_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.90 (C-1), 165.91 (C-1″), 156.66 (C-7), 134.36 (C-2″), 131.19 (C-5″), 130.80 (C-4), 130.19 (C-5, 9), 128.27 (C-4″, 6″), 127.21 (C-3″, 7″), 114.05 (C-6, 8), 64.58 (OCH_2CH_2N), 56.83 (OCH_2CH_2N), 55.17 (C-2), 44.54 ($N(CH_3)_2$), 35.85 (C-3); MS (ESI): 357.1 $[M + H]^+$.

Synthesis of compound *N*-[*N*-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-L-phenylalaniol-*N*-oxide (Impurity H)

To a stirring solution of **Y101** (2.45 g, 5 mmol) in CH_2Cl_2 (100 mL) was added 3-chloroperbenzoic acid (mCPBA) (1.73 g, 10 mmol) at 30 °C, the reaction mixture was stirred for 2 h. The bulk of CH_2Cl_2 was removed in vacuo. The residue was diluted with ethyl acetate (200 mL), sequentially washed with water (200 mL) and brine (200 mL). The ethyl acetate layer was dried over $MgSO_4$, filtered, concentrated, and the crude material was purified by column chromatography ($CHCl_3$/MeOH, 40/1) to yield impurity **H** (2.35 g, 98.34-% purity) in 93-% yield. Mp: 218–220 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, $J = 8.5$ Hz, 1H, 1″-CONH), 8.13 (d, $J = 8.3$ Hz, 1H, 1-CONH), 7.82 (d, $J = 8.0$ Hz, 2H, H-3″, 7″), 7.51 (t, $J = 7.3$ Hz, 1H, H-5″), 7.44 (t, $J = 7.4$ Hz, 2H, H-6″, 4″), 7.26–7.15 (m, 6H, H-5, 9, 5′, 6′, 8′, 9′), 7.12 (t, $J = 6.8$ Hz, 1H, H-7′), 6.83 (d, $J = 8.6$ Hz, 2H, H-6, 8), 4.66 (td, $J = 9.0$, 5.5 Hz, 1H, H-2), 4.46–4.37 (m, 2H, OCH_2CH_2N), 3.94–3.82 (m, 1H, H-2′), 3.55 (d, $J = 4.2$ Hz, 2H, OCH_2CH_2N), 3.36–3.21 (m, 2H, H-1′), 3.10 (d, $J = 5.8$ Hz, 6H, $N(CH_3)_2$), 3.02–2.80 (m, 3H, H-3a, 3′a, 3b), 2.67 (dd, $J = 13.6$, 7.8 Hz, 1H, H-3′b); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.05 (C-1), 166.12 (C-1″), 156.33 (C-7), 139.09 (C-4′), 134.13 (C-2″), 131.28 (C-5″), 130.85 (C-4), 130.32 (C-5, 9), 129.24 (C-5′, 9′), 128.19 (C-4″, 6″), 128.06 (C-6′, 8′), 127.47 (C-3″, 7″), 125.88 (C-7′), 114.15 (C-6, 8), 68.26 (OCH_2CH_2N), 62.10 (C-1′,

$O\underline{C}H_2CH_2N$), 59.14 ($N(\underline{C}H_3)_2$), 55.17 (C-2), 52.54 (C-2′), 36.48 (C-3, 3′); MS (ESI): 506.2 $[M + H]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-aminoethyl)-L-tyrosyl]-L-phenylalaninol (Impurity I)

*Synthesis of intermediate N-[N-benzoyl-O-(2-bromoethyl)-L-tyrosyl]-L-phenylalaninol (**10**)*

To a solution of **6** (4.2 g, 10 mmol) in anhydrous DMF (100 mL) were added 1,2-dibromoethane (18.8 g, 0.1 mol) and potassium carbonate (5.52 g, 40 mmol), the reaction mixture was stirred at 70 °C for 6 h. The reaction mixture was poured into water and extracted with ethyl acetate. The combined organic layer was washed with brine, and then dried over $MgSO_4$. Filtration and solvent evaporation gave a yellow solid. The compound was purified using column chromatography (silica gel, $CHCl_3$/MeOH, 100/1) to give intermediate **10** (4.46 g) in 85-% yield. Mp: 230–232 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, $J = 8.5$ Hz, 1H, 1″-CONH), 7.88 (d, $J = 8.3$ Hz, 1H, 1-CONH), 7.81–7.77 (m, 2H, H-3″, 7″), 7.54–7.49 (m, 1H, H-5″), 7.44 (t, $J = 7.5$ Hz, 2H, H-6″, 4″), 7.20 (ddd, $J = 20.4$, 11.7, 7.2 Hz, 6H, H-5, 9, 5′, 6′, 8′, 9′), 7.14–7.09 (m, 1H, H-7′), 6.82 (d, $J = 8.7$ Hz, 2H, H-6, 8), 4.81 (t, $J = 5.4$ Hz, 1H, OH), 4.62 (ddd, $J = 10.2$, 8.6, 4.5 Hz, 1H, H-2), 4.23 (t, $J = 5.1$ Hz, 2H, $OC\underline{H}_2CH_2Br$), 3.89 (td, $J = 7.9$, 2.3 Hz, 1H, H-2′), 3.75 (t, $J = 5.1$ Hz, 2H, $OCH_2C\underline{H}_2Br$), 3.30 (ddd, $J = 16.3$, 10.6, 4.5 Hz, 2H, H-1′), 2.99–2.83 (m, 3H, H-3a, 3′a, 3b), 2.66 (dd, $J = 13.6$, 8.0 Hz, 1H, H-3′b); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.03 (C-1), 166.13 (C-1″), 156.41 (C-7), 139.02 (C-4′), 134.11 (C-2″), 131.33 (C-5″), 130.88 (C-4), 130.31 (C-5, 9), 129.23 (C-5′, 9′), 128.23 (C-4″, 6″), 128.11 (C-6′, 8′), 127.44 (C-3″, 7″), 125.94 (C-7′), 114.21 (C-6, 8), 67.64 ($O\underline{C}H_2CH_2$ Br), 62.23 (C-1′), 55.06 (C-2), 52.49 (C-2′), 36.45 (C-3, 3′), 31.59 ($OCH_2\underline{C}H_2$ Br); MS (ESI): 1073.3$[2 M + Na]^+$.

*Synthesis of impurity **I***

$NH_3{\cdot}H_2O$ (1.4 g, 40 mmol) was added to the solution of intermediate **10** (1.05 g, 2 mmol) in tetrahydrofuran (THF; 50 mL). After stirring at 50 °C for 4 h, the bulk of THF was removed in vacuo, and partitioned between ethyl acetate and water. The organic phase was separated and washed with brine, and dried over $MgSO_4$. Filtration and solvent evaporation gave a buff oily compound. The compound was purified using column chromatography (silica gel, $CHCl_3$/MeOH, 25/1) to give impurity **I** (0.79 g, 99.60-% purity) in 85-% yield. Mp: 213–215 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (d, $J = 8.4$ Hz, 1H, 1″-CONH), 7.86 (d, $J = 8.2$ Hz, 1H, 1-CONH), 7.80 (d, $J = 7.3$ Hz, 2H, H-3″, 7″), 7.52 (t, $J = 7.1$ Hz, 1H, H-5″), 7.45 (t, $J = 7.3$ Hz, 2H, H-6″, 4″), 7.26–7.08 (m, 7H, H-5, 9, 5′, 6′, 8′, 9′, 7′), 6.80 (d, $J = 8.3$ Hz, 2H, H-6, 8), 4.63 (td, $J = 9.4$, 4.8 Hz, 1H, H-2), 3.91 (d, $J = 5.4$ Hz, 1H, H-2′), 3.84 (t, $J = 5.6$ Hz, 2H, $OC\underline{H}_2CH_2N$), 3.32 (ddd, $J = 24.7$, 10.6, 5.5 Hz, 2H, H-1′), 2.92 (ddd, $J = 23.7$, 13.7, 6.9 Hz, 3H, H-3a, 3′a, 3b), 2.82 (t, $J = 5.8$ Hz, 2H, $OCH_2C\underline{H}_2N$), 2.67 (dd, $J = 13.5$, 8.0 Hz, 1H, H-3′b); ^{13}C NMR

(101 MHz, DMSO-d_6) δ 170.96 (C-1), 166.03 (C-1″), 157.11 (C-7), 138.96 (C-4′), 134.09 (C-2″), 131.22 (C-5″), 130.15 (C-4), 130.12 (C-5, 9), 129.15 (C-5′, 9′), 128.14 (C-4″, 6″), 128.02 (C-6′, 8′), 127.36 (C-3″, 7″), 125.84 (C-7′), 113.98 (C-6, 8), 69.91 ($O\underline{C}H_2CH_2N$), 62.17 (C-1′), 55.04 (C-2), 52.43 (C-2′), 40.91 ($OCH_2\underline{C}H_2N$), 36.41 (C-3, 3′); MS (ESI): 462.3 $[M + H]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-methylaminoethyl)-L-tyrosyl]-L-phenylalaninol (Impurity K)

NH_2CH_3 (1.24 g, 40 mmol) was added to the solution of intermediate **10** (1.05 g, 2 mmol) in THF (50 mL). After stirring at 50 °C for 4 h, the bulk of THF was removed in vacuo, and partitioned between ethyl acetate and water. The organic phase was separated and washed with brine, and dried over $MgSO_4$. Filtration and solvent evaporation gave a buff oily compound. The compound was purified using column chromatography (silica gel, $CHCl_3$/MeOH, 25/1) to give impurity **K** (0.86 g, 99.77-% purity) in 90-% yield. Mp: 223-225 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, $J = 8.5$ Hz, 1H, 1″-CONH), 7.88 (d, $J = 8.3$ Hz, 1H, 1-CONH), 7.83–7.74 (m, 2H, H-3″, 7″), 7.56–7.49 (m, 1H, H-5″), 7.48–7.40 (m, 2H, H-6″, 4″), 7.25–7.16 (m, 6H, H-5, 9, 5′, 6′, 8′, 9′), 7.14 (dd, $J = 5.3$, 3.4 Hz, 1H, H-7′), 6.80 (d, $J = 8.7$ Hz, 2H, H-6, 8), 4.82 (s, 1H, OH), 4.62 (td, $J = 10.0$, 4.6 Hz, 1H, H-2), 3.98–3.83 (m, 3H, H-2′, $OC\underline{H}_2CH_2N$), 3.31 (dd, $J = 14.4$, 4.9 Hz, 2H, H-1′), 3.00–2.82 (m, 3H, H-3a, 3′a, 3b), 2.77 (t, $J = 5.6$ Hz, 2H, $OCH_2C\underline{H}_2N$), 2.66 (dd, $J = 13.7$, 8.0 Hz, 1H, H-3′b), 2.30 (s, 3H, $C\underline{H}_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.08 (C-1), 166.13 (C-1″), 157.08 (C-7), 139.02 (C-4′), 134.10 (C-2″), 131.34 (C-5″), 130.27 (C-4), 130.22 (C-5, 9), 129.24 (C-5′, 9′), 128.25 (C-4″, 6″), 128.12 (C-6′, 8′), 127.45 (C-3″, 7″), 125.95 (C-7′), 114.02 (C-6, 8), 66.89 ($O\underline{C}H_2CH_2N$), 62.25 (C-1′), 55.12 (C-2), 52.51 (C-2′), 50.26 ($OCH_2\underline{C}H_2N$), 36.47 (C-3, 3′), 36.10 ($\underline{C}H_3$); MS (ESI): 476.2$[M + H]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-methylaminoethyl)-L-tyrosyl]-*O*-benzoyl-L-phenylalaninol (Impurity O)

Intermediate **10** (1.05 g, 2 mmol) was dissolved in 20 mL of anhydrous pyridine under argon atmosphere, followed by addition of benzoyl chloride (0.35 g, 2.5 mmol) and 4-dimethylaminopyridine (DMAP; 0.1 g, 0.82 mmol). The reaction mixture was stirred at 50 °C for 4 h. Ethyl acetate and water were charged, and the mixture was stirred for 30 min. The organic layer was washed with brine, and then dried over $MgSO_4$, filtered and concentrated. The crude material was dissolved in 100 mL of THF and reacted with NH_2CH_3 (1.24 g, 40 mmol) at 50 °C for 4 h. The bulk of THF was removed in vacuo, and partitioned between ethyl acetate and water. The organic phase was separated and washed with brine, and dried over $MgSO_4$. Filtration and solvent evaporation gave a buff oily compound. The compound was purified using column chromatography (silica gel, $CHCl_3$/MeOH, 60/1) to give impurity **O** (0.81 g, 98.50-% purity) in 70-% yield. Mp: 208–210 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51 (d, $J = 8.4$ Hz, 1H, 1″-CONH), 8.33 (d, $J = 8.3$ Hz, 1H, 1-CONH), 7.99 (d, $J = 7.3$ Hz, 2H, H-3‴, 7‴), 7.80 (d,

$J = 7.3$ Hz, 2H, H-3″, 7″), 7.66 (t, $J = 7.4$ Hz, 1H, H-5‴), 7.51 (t, $J = 7.8$ Hz, 3H, H-4‴, 6‴, 5″), 7.42 (t, $J = 7.6$ Hz, 2H, H-4″, 6″), 7.28 (d, $J = 7.2$ Hz, 2H, H-5, 9), 7.24 (t, $J = 7.5$ Hz, 2H, H-5′, 9′), 7.17 (d, $J = 8.5$ Hz, 3H, H-6′, 7′, 8′), 6.76 (d, $J = 8.5$ Hz, 2H, H-6, 8), 4.66 (dd, $J = 14.6$, 8.7 Hz, 1H, H-2), 4.40– 4.31 (m, 1H, H-2′), 4.26 (dd, $J = 11.0$, 4.3 Hz, 1H, H-1′a), 4.15 (dd, $J = 10.9$, 6.3 Hz, 1H, H-1′b), 3.95 (t, $J = 5.4$ Hz, 2H, $OC\underline{H}_2CH_2N$), 2.90 (t, $J = 5.4$ Hz, 6H, H-3, 3′, $OCH_2C\underline{H}_2N$), 2.38 (s, 3H, $C\underline{H}_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.28 (C-1), 166.02 (C-1″), 165.59 (C-1‴), 156.77 (C-7), 138.02 (C-4′), 134.03 (C-4), 133.32 (C-5‴), 131.20 (C-2″), 130.30 (C-5″), 130.05 (C-5, 9), 129.55 (C-2‴), 129.30 (C-3‴, 7‴), 129.11 (C-5′, 9′), 128.61 (C-4‴, 6‴), 128.23 (C-6′, 8′), 128.10 (C-4″, 6″), 127.38 (C-3″, 7″), 126.22 (C-7′), 113.99 (C-6, 8), 65.74 ($O\underline{C}H_2CH_2N$), 65.49 (C-1′), 55.11 (C-2), 49.36 (C-2′), 49.22 ($OCH_2\underline{C}H_2N$), 36.62 (C-3), 36.47 (C-3′), 35.07 ($\underline{C}H_3$); MS (ESI): 580.3 $[M + H]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-*O*-acetyl-L-phenylalaninol (Impurity N)

To a solution of **Y101** (4.9 g, 10 mmol) and DMAP (100 mg) in CH_2Cl_2 (150 mL) was added acetic anhydride (1.23 g, 12 mmol) at 30 °C. The mixture was stirred for 3 h,then adjusted to pH 8 with saturated $NaHCO_3$ solution. The reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, and then dried over $MgSO_4$. Filtration and solvent evaporation gave a buff solid. The compound was recrystallized from ethyl acetate to afford impurity **N** (4.52 g, 99.22-% purity) in 85-% yield. Mp: 184–186 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, $J = 8.5$ Hz, 1H, 1″-CONH), 8.11 (d, $J = 8.5$ Hz, 1H, 1-CONH), 7.80 (dd, $J = 8.3$, 1.3 Hz, 2H, H-3″, 7″), 7.52 (ddd, $J = 6.5$, 3.8, 1.4 Hz, 1H, H-5″), 7.48–7.41 (m, 2H, H-6″, 4″), 7.25–7.18 (m, 6H, H-5, 9, 5′, 6′, 8′, 9′), 7.18–7.12 (m, 1H, H-7′), 6.81 (d, $J = 8.7$ Hz, 2H, H-6, 8), 4.61 (td, $J = 9.1$, 5.4 Hz, 1H, H-2), 4.18 (dd, $J = 12.4$, 7.3 Hz, 1H, H-2′), 4.01 (dd, $J = 11.0$, 4.8 Hz, 1H, H-1′a), 3.96 (t, $J = 5.9$ Hz, 2H, $OC\underline{H}_2CH_2N$), 3.86 (dd, $J = 11.0$, 6.7 Hz, 1H, H-1′b), 2.95–2.85 (m, 2H, H-3a, 3′a), 2.78 (dd, $J = 6.9$, 4.5 Hz, 2H, H-3b, 3′b), 2.56 (t, $J = 5.9$ Hz, 2H, $OCH_2C\underline{H}_2N$), 2.18 (s, 6H, $N(C\underline{H}_3)_2$), 1.98 (s, 3H, $CO\underline{CH}_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.29 (C-1), 170.30 ($\underline{C}OCH_3$), 166.11 (C-1‴), 156.98 (C-7), 138.00 (C-4′), 134.06 (C-2″), 131.29 (C-5″), 130.16 (C-4), 130.12 (C-5, 9), 129.14 (C-5′, 9′), 128.23 (C-4″, 6″), 128.18 (C-6′, 8′), 127.43 (C-3″, 7″), 126.23 (C-7′), 114.00 (C-6, 8), 65.60 ($OCH_2\underline{C}H_2N$), 64.63 (C-1′), 57.74 ($O\underline{C}H_2CH_2N$), 55.15 (C-2), 49.11 (C-2′), 45.55 ($N(\underline{C}H_3)_2$), 36.59 (C-3), 36.45 (C-3′), 20.61 ($CO\underline{C}H_3$); MS (ESI): 532.3 $[M + H]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-*O*-acetyl-L-phenylalaninol-*N*-oxide (Impurity J)

To a stirring solution of impurity **N** (2.66 g, 5 mmol) in CH_2Cl_2 (100 mL) was added mCPBA (1.73 g, 10 mmol) at 30 °C, the reaction mixture was stirred for 2 h. The bulk of CH_2Cl_2 was removed in vacuo. The residue was diluted with ethyl acetate (200 mL), and sequentially washed with water (200 mL) and brine

(200 mL). The ethyl acetate layer was dried over $MgSO_4$, filtered, concentrated, and the crude material was purified by column chromatography ($CHCl_3$/MeOH, 40/1) to yield impurity **J** (2.46 g, 98.16-% purity) in 90-% yield. Mp: 174–176 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (d, J = 8.4 Hz, 1H, 1″-CONH), 8.23 (d, J = 8.3 Hz, 1H, 1-CONH), 7.85–7.78 (m, 2H, H-3″, 7″), 7.51 (ddd, J = 6.4, 3.8, 1.4 Hz, 1H, H-5″), 7.48–7.40 (m, 2H, H-6″, 4″)), 7.28–7.20 (m, 6H, H-5, 9, 5′, 6′, 8′, 9′), 7.18–7.12 (m, 1H, H-7′), 6.85 (d, J = 8.7 Hz, 2H, H-6, 8), 4.68–4.57 (m, 1H, H-2), 4.42 (t, J = 4.8 Hz, 2H, $OC\underline{H}_2CH_2N$), 4.18 (dd, J = 12.6, 7.1 Hz, 1H, H-2′), 4.01 (dd, J = 11.0, 4.8 Hz, 1H, H-1′a), 3.86 (dd, J = 11.0, 6.7 Hz, 1H, H-1′b), 3.52 (t, J = 4.4 Hz, 2H, $OCH_2C\underline{H}_2N$), 3.08 (s, 6H, $N(C\underline{H}_3)_2$), 2.92 (d, J = 7.9 Hz, 2H, H-3a, 3′a), 2.83–2.71 (m, 2H, H-3b, 3′b), 1.98 (s, 3H, $COC\underline{H}_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.36 (C-1), 170.32 ($\underline{C}OCH_3$), 166.16 (C-1″), 156.37 (C-7), 138.03 (C-4′), 134.09 (C-2″), 131.30 (C-5″), 130.76 (C-4), 130.28 (C-5, 9), 129.16 (C-5′, 9′), 128.24 (C-4″, 6″), 128.19 (C-6′, 8′), 127.47 (C-3″, 7″), 126.24 (C-7′), 114.14 (C-6, 8), 68.27 ($OCH_2\underline{C}H_2N$), 64.65 (C-1′), 62.04 ($O\underline{C}H_2CH_2N$), 59.19 ($N(\underline{C}H_3)_2$), 55.19 (C-2), 49.12 (C-2′), 36.59 (C-3), 36.41 (C-3′), 20.64 ($CO\underline{C}H_3$); MS (ESI): 570.2 $[M + Na]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-acetyl-L-tyrosyl]-*O*-acetyl-L-phenylalaninol (impurity L)

Acetic anhydride (2.25 g, 22 mmol) was added to a mixture of compound **6** (4.2 g, 10 mmol) and DMAP (100 mg) in DMF (50 mL). After stirring at 30 °C for 4 h, the mixture was acidified to pH 8 with saturated $NaHCO_3$ solution, and partitioned between ethyl acetate and water. The organic phase was separated and sequentially washed with brine, and dried over $MgSO_4$. Filtration and solvent evaporation gave a residue which was recrystallized from MeOH-$CHCl_3$ to afford target impurity **L** (3.92 g, 99.17-% purity) in 78-% yield. Mp: 220–222 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (d, J = 8.5 Hz, 1H, 1″-CONH), 8.16 (d, J = 8.4 Hz, 1H, 1-CONH), 7.80 (dd, J = 5.2, 3.3 Hz, 2H, H-3″, 7″), 7.56–7.49 (m, 1H, H-5″), 7.48–7.42 (m, 2H, H-6″, 4″), 7.34 (d, J = 8.6 Hz, 2H, H-5′, 9′), 7.26–7.17 (m, 5H, H-5, 9, 6′, 7′, 8′), 7.01 (d, J = 8.5 Hz, 2H, H-6, 8), 4.68 (td, J = 9.0, 5.7 Hz, 1H, H-2), 4.20 (dd, J = 12.5, 7.2 Hz, 1H, H-2′), 4.04 (dd, J = 11.0, 4.8 Hz, 1H, H-1′a), 3.88 (dd, J = 11.0, 6.8 Hz, 1H, H-1′b), 3.05–2.92 (m, 2H, H-3a, 3′a), 2.80 (dd, J = 6.8, 5.0 Hz, 2H, H-3b, 3′b), 2.22 (s, 3H, $COC\underline{H}_3$), 1.99 (s, 3H, $COC\underline{H}_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.15 (C-1), 170.30 ($\underline{C}OCH_3$), 169.16 ($\underline{C}OCH_3$), 166.22 (C-1″), 148.93 (C-7), 137.98 (C-4′), 135.81 (C-2″), 134.02 (C-5″), 131.31 (C-5, 9), 130.06 (C-5′, 9′), 129.15 (C-4), 128.23 (C-4″, 6″), 128.18 (C-6′, 8′), 127.43 (C-3″, 7″), 126.23 (C-7′), 121.33 (C-6, 8), 64.60 (C-1′), 54.82 (C-2), 49.17 (C-2′), 36.58 (C-3, C-3′), 20.84 ($CO\underline{C}H_3$), 20.62 ($CO\underline{C}H_3$); MS (ESI): 525.2 $[M + Na]^+$.

Synthesis of compound *N*-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-*O*-benzoyl-L-phenylalaninol-*N*-oxide (impurity M)

To a stirring solution of impurity **F** (0.47 g, 0.8 mmol) in CH_2Cl_2 (40 mL) was added mCPBA (0.26 g, 1.5 mmol) at 30 °C, the reaction mixture was stirred for

2 h. The bulk of CH_2Cl_2 was removed in vacuo. The residue was diluted with ethyl acetate (100 mL), and sequentially washed with water (100 mL) and brine (100 mL). The ethyl acetate layer was dried over $MgSO_4$, filtered, concentrated, and the crude material was purified by column chromatography ($CHCl_3$/MeOH, 40/1) to yield impurity **M** (0.44 g, 98.73-% purity) in 90-% yield. Mp: 166–168 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (d, J = 8.4 Hz, 1H, 1″-CONH), 8.37 (d, J = 8.3 Hz, 1H, 1-CONH), 7.98 (d, J = 7.5 Hz, 2H, H-3‴, 7‴), 7.79 (d, J = 7.5 Hz, 2H, H-3″, 7″), 7.65 (t, J = 7.3 Hz, 1H, H-5‴), 7.54–7.47 (m, 3H, H-4‴, 6‴, 5″), 7.42 (t, J = 7.6 Hz, 2H, H-4″, 6″), 7.31–7.21 (m, 4H, H-5, 9, 5′, 9′), 7.17 (dd, J = 12.2, 7.7 Hz, 3H, H-6′, 7′, 8′), 6.78 (d, J = 8.4 Hz, 2H, H-6, 8), 4.67 (dd, J = 14.9, 8.1 Hz, 1H, H-2), 4.38 (t, J = 4.5 Hz, 3H, H-2′, OC$\underline{H}_2$C$\underline{H}_2$N), 4.26 (dd, J = 10.9, 4.2 Hz, 1H, H-1′a), 4.14 (dd, J = 10.9, 6.4 Hz, 1H, H-1′b), 3.45 (s, 2H, OCH$_2$C$\underline{H}_2$N), 3.03 (d, J = 2.9 Hz, 6H, N(C$\underline{H}_3$)$_2$), 2.89 (d, J = 5.7 Hz, 4H, H-3a, 3′a, 3b, 3′b); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.53 (C-1), 166.21 (C-1″), 165.70 (C-1‴), 156.37 (C-7), 138.11 (C-4′), 134.08 (C-4), 133.47 (C-5‴), 131.33 (C-2″), 130.70 (C-5″), 130.24 (C-5, 9), 129.61 (C-2‴), 129.42 (C-3‴, 7‴), 129.24 (C-5′, 9′), 128.74 (C-4‴′, 6‴), 128.36 (C-6′, 8′), 128.22 (C-4″, 6″), 127.52 (C-3″, 7″), 126.35 (C-7′), 114.10 (C-6, 8), 68.29 (OCH$_2$$\underline{C}H_2$N), 65.55 (C-1′), 62.02 (O$\underline{C}$H$_2$CH$_2$N), 59.31 (N($\underline{C}$H$_3$)$_2$), 55.21 (C-2), 49.26 (C-2′), 36.68 (C-3), 36.52 (C-3′); MS (ESI):610.2 $[M + H]^+$.

Results and discussion

To identify the structure of a low-level unknown impurity, LC/MS/high-resolution MS (HRMS) and tandem MS (MS/MS) were used to determine their molecular weight (MW), elemental composition, and fragmentation patterns. On the basis of the MS data and knowledge of the process chemistry, nine possible structures (Table 1) were assigned for the impurities. MS alone without authentic standards can never be sufficient for the unequivocal confirmation of the structure of impurities. Thus, the chemical synthesis of nine impurities was carried out, and their structures were elucidated on the basis of NMR and MS. The structures of the nine primary impurities in API were unequivocally confirmed by comparing the HPLC retention time, the ultraviolet (UV) spectrum profile, and the mass spectrum with that of the synthetic standards.

Impurity synthesis

The quantity of these impurities required for to confirm their structure, for validation, and for use as analytical standards was substantial. Thus, a synthetic route was required for each impurity which could be used to cross-validate the impurity versus the postulated structure and against the impurity seen in the analytical method (allowing for confirmation of retention time, relative response factor, etc.).

The impurity **G** was prepared from L-tyrosine methyl ester hydrochloride (**8**) in three steps, as shown in Scheme 2. The reaction of benzoic acid with **8** gave the

Table 1 Structures of impurities **G–O**

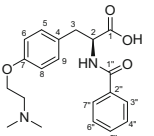
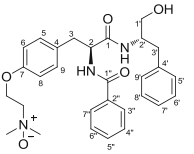
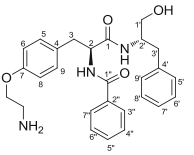
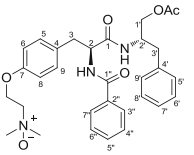
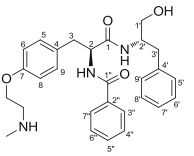
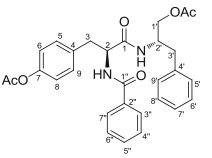
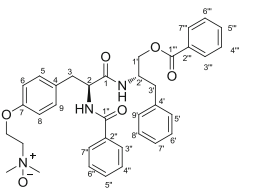
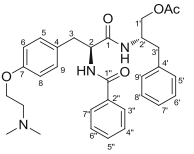
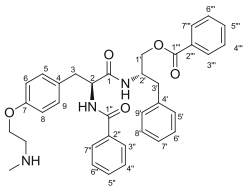
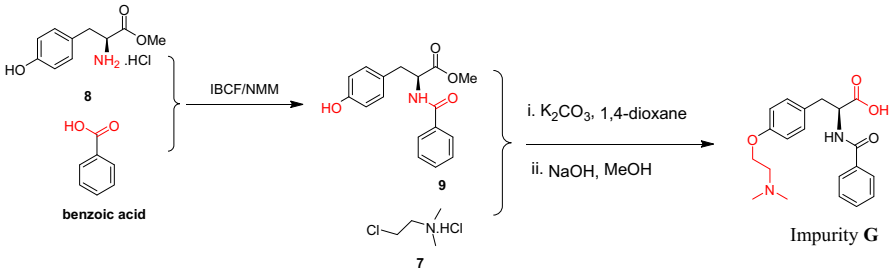
Entry	Name	Structure	Relative retention time[a]
1	Impurity-**G**		0.30
2	Impurity-**H**		0.45
3	Impurity-**I**		0.57
4	Impurity-**J**		0.65
5	Impurity-**K**		0.70
6	Impurity-**L**		1.20
7	Impurity-**M**		1.57
8	Impurity-**N**		1.59

Table 1 continued

Entry	Name	Structure	Relative retention time[a]
9	Impurity-**O**		3.16

[a] Agilent 1100 HPLC; column: Phenomenex Gemini-NX C_{18} (250 mm × 4.6 mm, 5 μm); column temperature: 30 °C; mobile phase: MeOH–water (65:35) containing 0.04-% triethylamine, adjusted to pH 9.0 with phosphoric acid; flow rate: 1.0 mL/min; injection volume: 10 μL; wavelength for UV detection: 226 nm

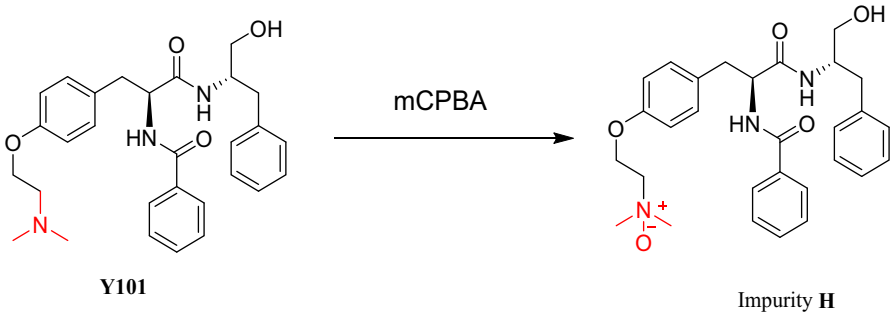


Scheme 2 Synthesis of impurity **G**

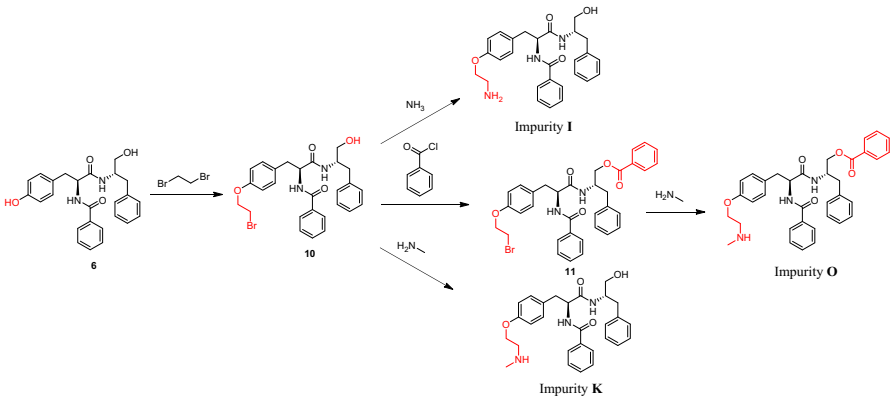
compound **9** [8–12]. Alkylation of the hydroxyl group of compound **9** with dimethylaminoethyl chloride and followed by hydrolysis of the ester bond with NaOH furnished impurity **G.**

As shown in Scheme 3, impurity **H** was obtained by reaction of **Y101** with mCPBA in CH_2Cl_2 at 30 °C [13, 14].

As outlined in Scheme 4, a straightforward and efficient methodology for the synthesis of impurities **I**, **K**, and **O** was developed. Compound **10** was the key



Scheme 3 Synthesis of impurity **H**



Scheme 4 Synthesis of impurities **I, K,** and **O**

intermediate for synthesis of the impurities investigated. Treating compound **6** with excessive amounts of 1,2-dibromoethane at 70 °C for 4 h in anhydrous DMF catalyzed by potassium carbonate yielded compound **10**. Reactions of **10** with excess ammonia-water and methylamine in DMF at 50 °C for 4 h yielded impurities **I** and **K**. In addition, derivative **11** was prepared by treating compound **10** in situ with benzoyl chloride at 50 °C for 4 h in pyridine catalyzed by DMAP. Finally, impurity **O** was synthesized by treatment of compound **11** with excess methylamine [15, 16].

The above study indicated that when impurities **I** and **K** were synthesized by treating compound **10** with excess ammonia-water and methylamine in DMF catalyzed by potassium carbonate, large amounts of by-product (**Y101**) were observed. A hypothesis mechanism was shown in Fig. 2. From these results, the K_2CO_3 was removed, and the reaction solvent was changed to THF. Using this newly developed synthetic method, impurities **I** and **K** were synthesized in excellent yield.

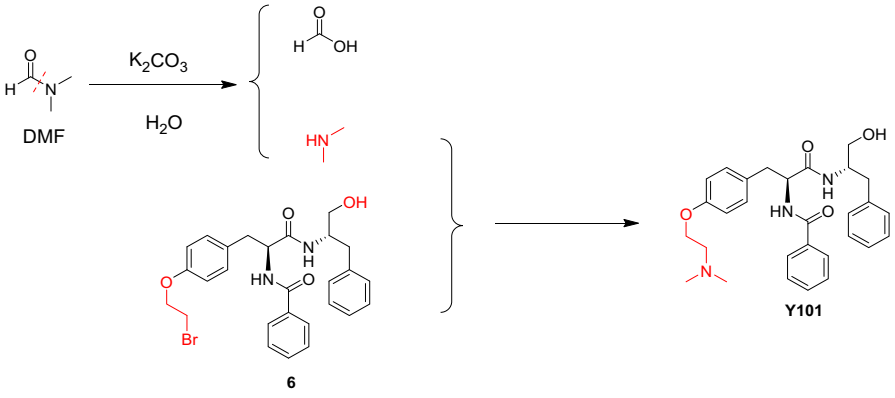
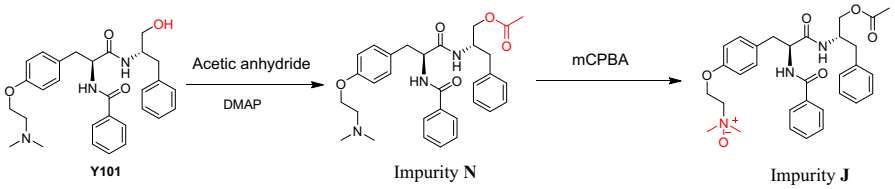


Fig. 2 Formation of by-product (**Y101**)

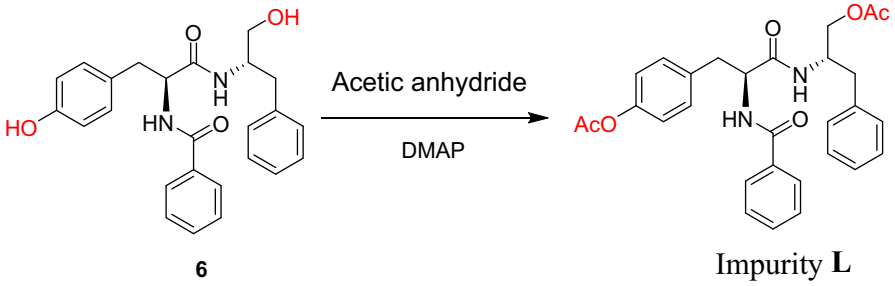


Scheme 5 Synthesis of impurities **N** and **J**

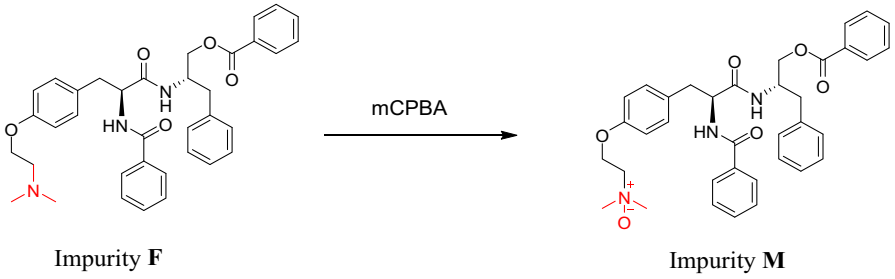
Impurities **N** and **J** could be synthesized from the same starting material (**Y101**), as shown in Scheme 5. Impurity **N** was obtained by reaction of **Y101** with acetic anhydride in the presence of DMAP in CH_2Cl_2 at 30 °C [17–19]. Treatment of impurity **N** with mCPBA in CH_2Cl_2 gave impurity **J**.

Impurity **L** was synthesized by reaction of compound **6** and acetic anhydride in the presence of DMAP in DMF at 30 °C (Scheme 6).

A synthetic protocol for impurity **M** was shown in Scheme 7. Impurity **F** was identified as a starting material, and impurity **M** was synthesized in the presence of mCPBA in CH_2Cl_2.



Scheme 6 Synthesis of impurity **L**



Scheme 7 Synthesis of impurity **M**

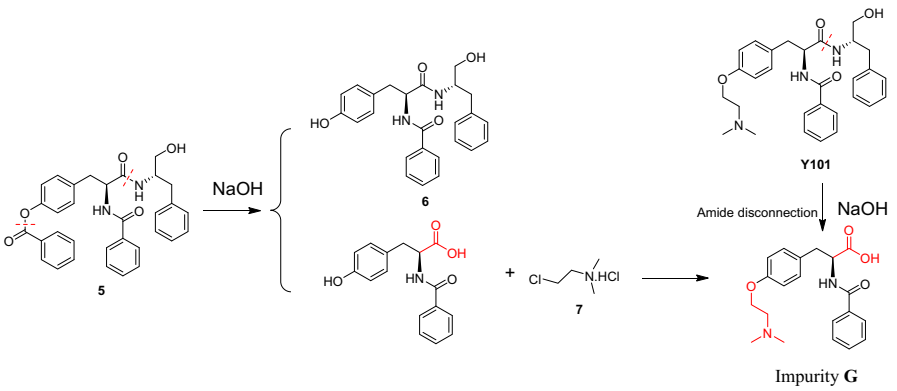


Fig. 3 Possible pathway to impurity **G**

Plausible pathways to impurities

*Impurity **G***

Impurity **G** was observed in the range of 0.02–0.05 % in the laboratory experimental study. This impurity was identified as the hydrolysis product of **Y101**, and might be due to the carryover of NaOH to the reaction of **5** to **6** and workup of the final step (Fig. 3) [7, 20].

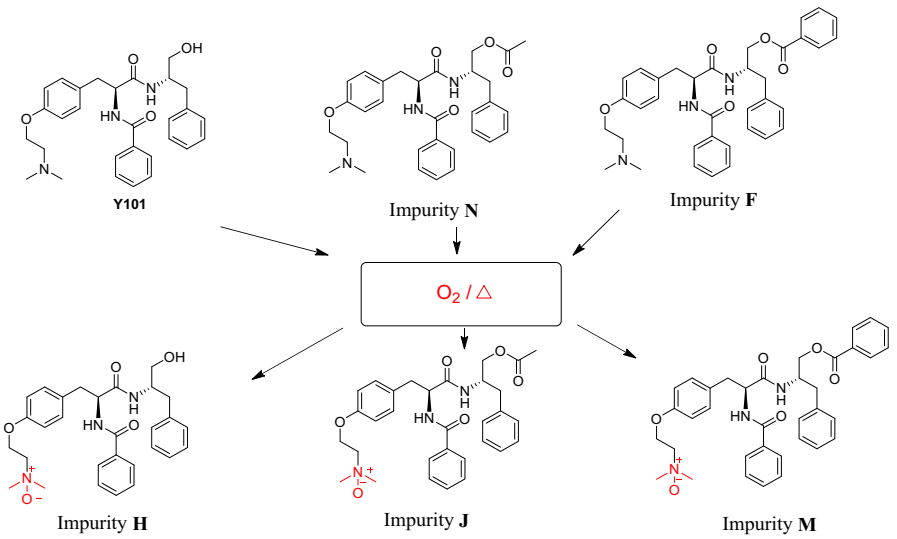


Fig. 4 Possible pathway to impurities **H**, **J**, and **M**

*Impurities **H**, **J**, and **M***

The process impurities **H**, **J**, and **M** were observed in the range of 0.03–0.07 %. Impurity **H** was the oxidation product of **Y101** and might be formed during the drying process of the drug substance (Fig. 4). Impurities **J** and **M** were structural analogues of impurity **H**, where the hydroxyl group had been replaced by the corresponding acetate and benzoate esters. Impurity **J** might be generated due to the presence of trace impurity **N** during the reaction of **6** to **Y101** (Fig. 4). The synthetic pathway for impurity **M** was shown in Fig. 4; similarly, impurity **M** might be formed by oxidation reaction of impurity **F** [20].

*Impurities **I**, **K**, and **O***

Impurity **K** was found to be present at a level greater than 0.10 % in the crude product of **Y101**, and upon being isolated from the filtrate of **Y101** recrystallization, the structure was confirmed by NMR and MS. This impurity was probably formed due to small amounts of 2-methylaminoethyl chloride hydrochloride presented in 2-dimethylaminoethyl chloride hydrochloride **7** (Fig. 5) [21, 22]. Impurity **I** presented at lower concentrations, and was also caused by the presence of an other impurity (2-aminoethyl chloride hydrochloride) in compound **7**. Impurity **O** was likely introduced by reaction of impurity **B** with impurity **K** (Fig. 5) [20].

Impurities L and N

The process impurities **L** and **N** were observed in the range of 0.04–0.06 %. Impurity **L** was identified as the acetic ester of compound **6** (the intermediate of **Y101** shown in Scheme 1). Impurity **N** was a homologous series of **Y101** acetic ester, which was similar to **Y101**. The two impurities were believed to arise from related acetic acid in the purification step of the alkylation reaction. As shown in Fig. 6, the acetic ester groups in impurities **L** and **N** can be traced back to ethyl acetate, which was the extraction solvent of the alkylation reaction (Scheme 1) [7,

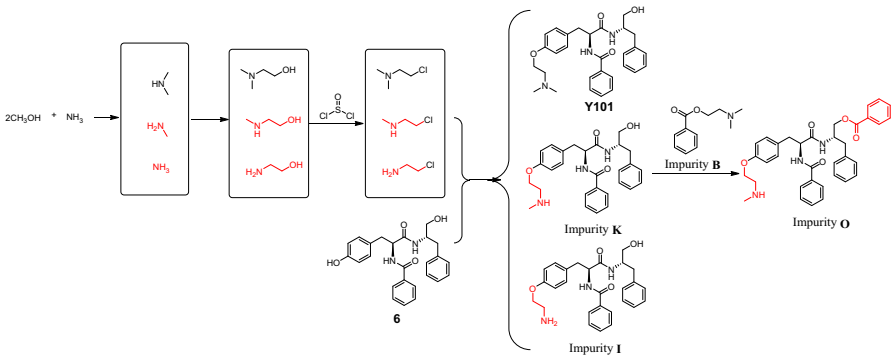


Fig. 5 Possible pathway to impurities **I**, **K**, and **O**

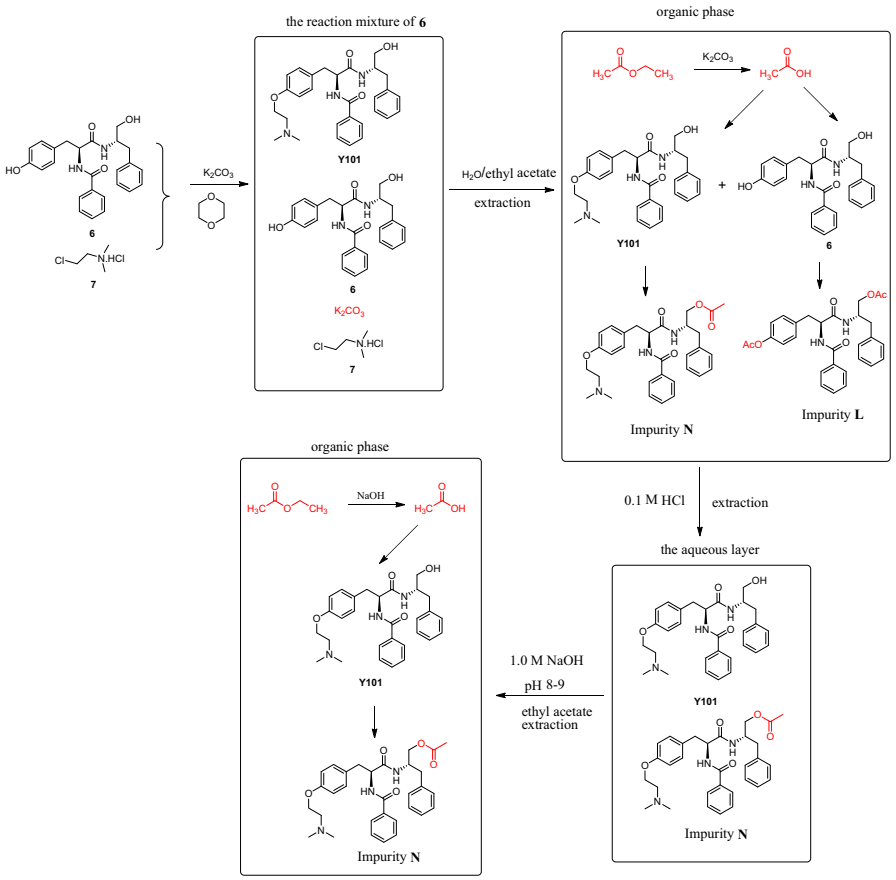


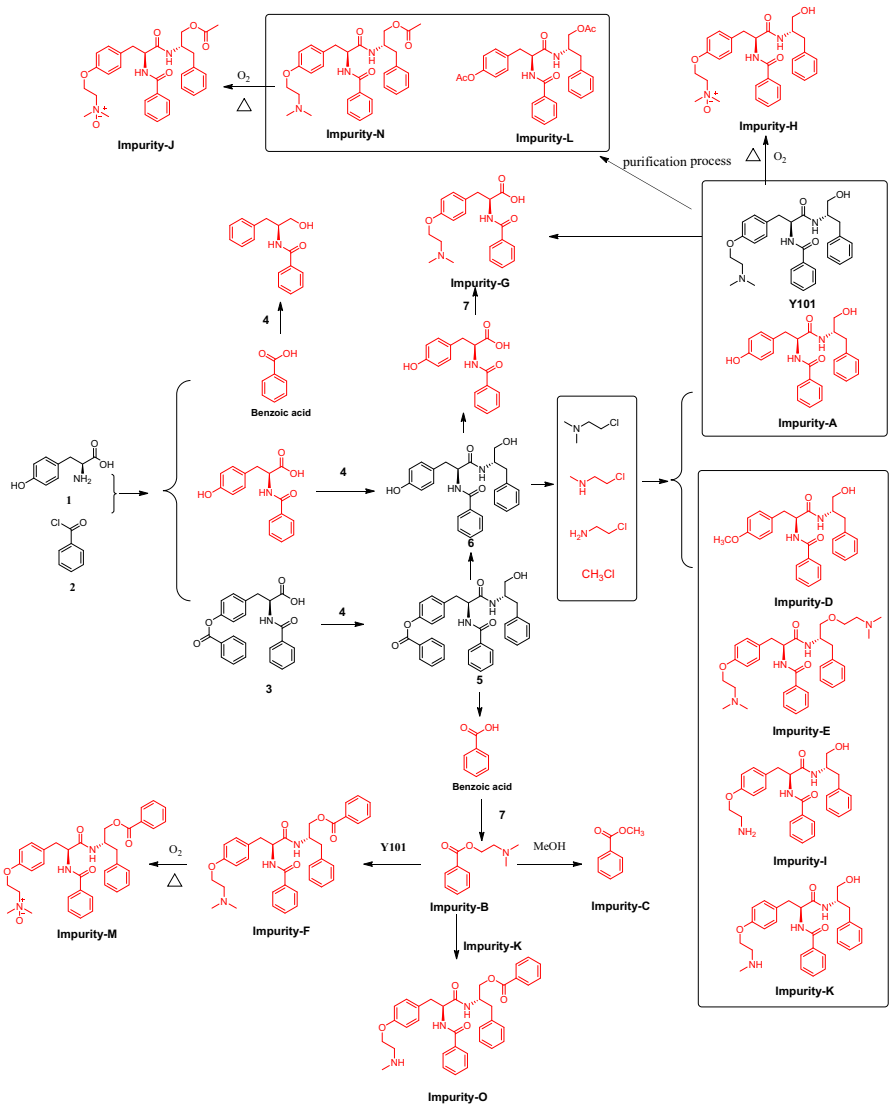
Fig. 6 Possible pathway to impurities **L** and **N**

20]. It is generally believed that there are small amounts of acetic acid in the extraction solution when ethyl acetate was poured into the alkaline aqueous solution of K_2CO_3 and NaOH.

Impurity control

Based on the initial input on potential impurities by the process research [20], impurity tracking from starting materials, through intermediates and finally to the API manufacturing process was carried out (Scheme 8). The potential impurities of each stage postulated during the process research were used as the basis for quality control.

Impurity **G** was the degradation product, and it could be controlled to a negligible level during the final step. This was achieved by purification in ethyl acetate.



Scheme 8 Impurity tracking of **Y101** process

Impurity **H** was a thermodynamically controlled impurity, and its rate of formation increased exponentially along with an increase in temperature. By conducting the drying temperature below 50 °C, the impurity **H** can be controlled to a level of 0.02 % in API.

Following Scheme 8, starting material **7** was known to contain small amounts of 2-methylaminoethyl chloride hydrochloride and a small quantity of 2-aminoethyl chloride hydrochloride as impurities. These compounds followed the same sequence as described for the API synthesis (Scheme 1) and leaded to the formation of

impurities **K** and **I**. Impurities **K** and **I** were analogues of **Y101**, and hence, the downstream process was unsuccessful at eliminating these impurities, because of their similar structural properties. Due to the difficult removal of impurities **K** and **I** from **Y101**, we controlled the potential impurities of 2-methylaminoethyl chloride hydrochloride and 2-aminoethyl chloride hydrochloride in **7**, and these impurities are removed by methanol purification. When **Y101** was obtained by reaction of **6** with the purified **7**, impurities **I** and **K** were reduced to lower than 0.06 %.

Impurity **O** was likely introduced by reaction of impurity **B** with impurity **K**, and was controlled to a non-detectable level in **Y101**, when the impurities **B** and **K** were reduced to lower than 0.1 %.

Impurities **J**, **L**, and **N** were process impurities and appeared in **Y101** due to carryover of acetic acid in the extraction solution, when ethyl acetate was poured into the alkaline aqueous solution of K_2CO_3 and NaOH. The formation of impurities **L** and **N** depended on the basicity of the reaction mixture. Furthermore, the formation of impurities **J**, **L**, and **N** were controlled to less than 0.04 % when the extraction solution pH was 8–9, and they were removed to a negligible level during the purification of **Y101** from ethyl acetate.

Impurity **M** might be formed due to the presence of impurity **F** in **Y101**. During process optimization, we observed that when impurity **F** was reduced to lower than 0.1 % [20], impurity **M** was controlled to a non-detectable level in **Y101**.

Conclusions

The structures of novel potential impurities **G–O** and their origins of formation during the preparation of **Y101** were identified. The knowledge of the structures led to the development of a re-work protocol that purged out these impurities to level accepted by the ICH.

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