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Identification, Synthesis and Strategy for Minimization of Potential Impurities in the Preclinical Anti-HBV Drug **Y101**

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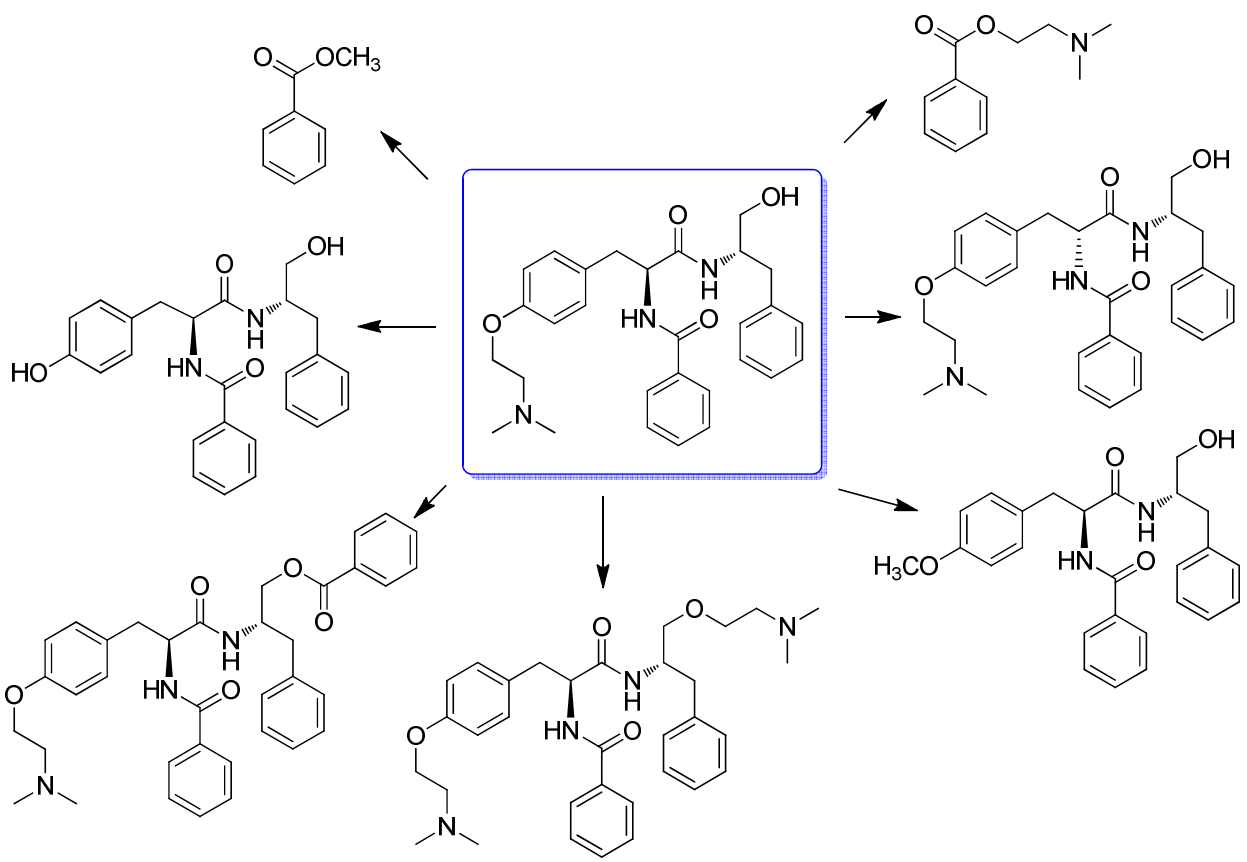


ABSTRACT: The identification of actual, potential and theoretical impurities of *N*-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-L-phenylalaninol (**Y101**), a preclinical anti-HBV drug, is described in this article. The impurities were monitored by HPLC and their structures were established on the basis of NMR, IR and MS. Most of the impurities were synthesized, and their assigned constitutions were confirmed by HPLC co-injection with an ordinary column (Phenomenex Gemini, 250 mm×4.6 mm, 5 μm) or a chiral column (DAICEL Chiralcel OD-H). According to the synthetic route, the origins of all of these related impurities were analyzed and some practical strategies were applied for minimizing these impurities to the level accepted by the International Conference on Harmonization (ICH) and therefore, these strategies can be well applied to the quality control in **Y101** clinical sample manufacture.

INTRODUCTION

N-[*N*-benzoyl-*O*-(2-dimethylaminoethyl)-L-tyrosyl]-L-phenylalaninol (**Y101**, Figure. 1) belongs to a new class of compound discovered for anti-HBV by our research group.[1] **Y101** can inhibit the secretion of HBsAg, HBeAg and the expression of HBV DNA in HepG2 2.2.15 cell line, which indicated that **Y101** had inhibitory effect on HBV. The preliminary study on the mechanism of anti-HBV effect of **Y101** showed that it could inhibit the expression of covalently closed circular DNA (cccDNA) rather than the HBV replication complexes (RCs) and DNA polymerase (DNAP). **Y101** might be a potential novel agent against HBV-infected hepatitis.[2a-d]

Y101 was indicated for the treatment of chronic hepatitis B associated with alanine aminotransferase (ALT) increased 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. The clinical trial application of anti-HBV drug **Y101** was submitted to China Food and Drug Administration (CFDA) at the end of 2012 (Handling No. CXHL1200877).

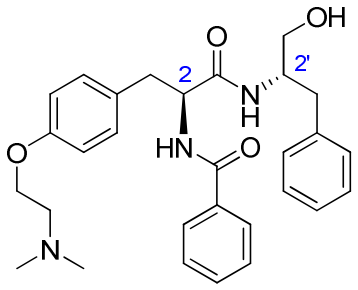
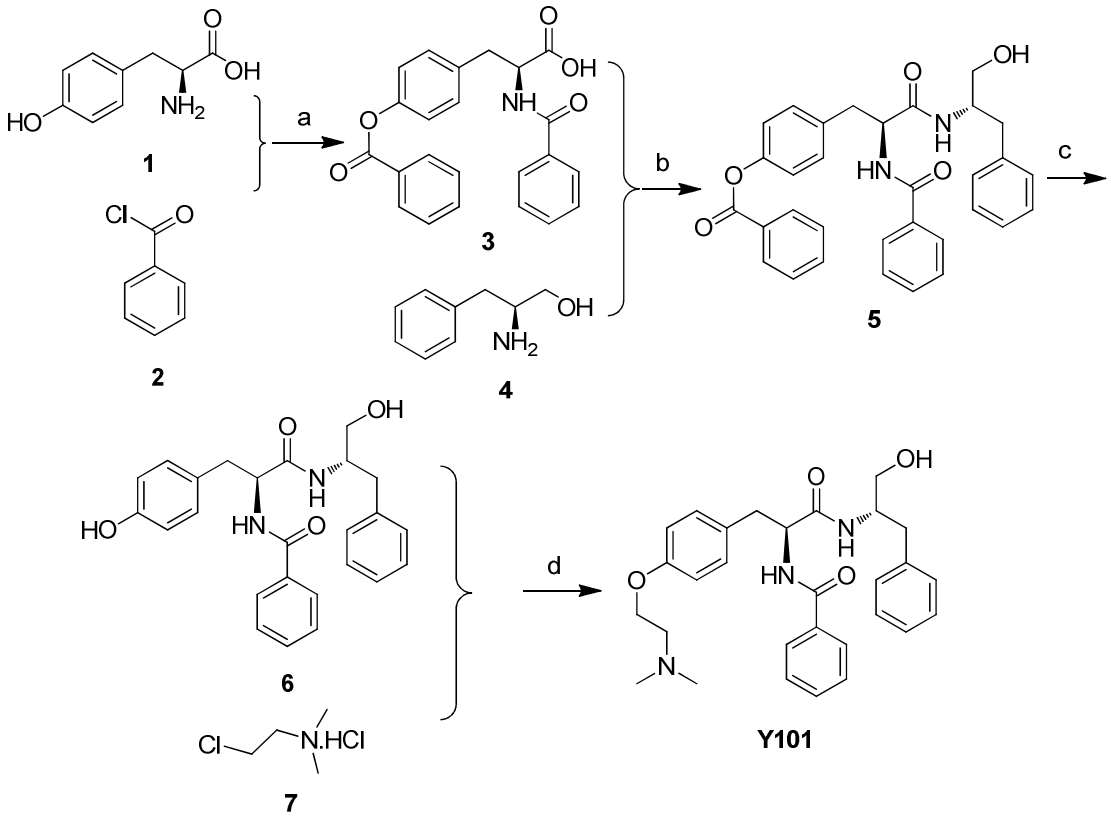


Figure 1. Structure of **Y101**.

The control of impurities observed in the active pharmaceutical ingredient (API) is critical in delivering an API of high quality. Thus, the detection, identification, quantification, and control of these impurities originating in the manufacturing process have become an important element of drug development in order to ensure product quality and ultimately patient safety. [3]

When **Y101** was synthesized following Scheme 1, a number of impurities were appearing in the drug substance. According to ICH guideline on impurities in new drug substance, impurities at levels greater than 0.10% or 1.0 mg per day intake (whichever is lower) should be identified for drugs with a maximum daily dose equal to or lesser than 2 g, and the identification should be attempted for those potential impurities that are expected to be unusually potent, producing toxic or pharmacologic effects at levels of not more than 0.1%.[3] Six impurities appearing in the sample of the crude drug substance were required to be identified. In this paper, the formation, identification, synthesis, characterization and strategy for controlling these impurities in **Y101** were described.

Scheme 1. The synthetic scheme of Y101[a]



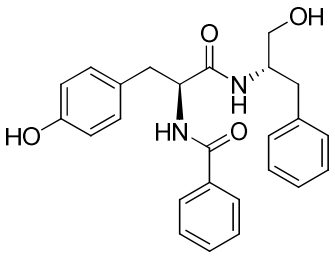
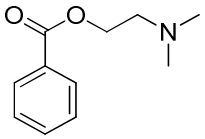
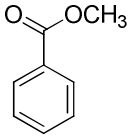
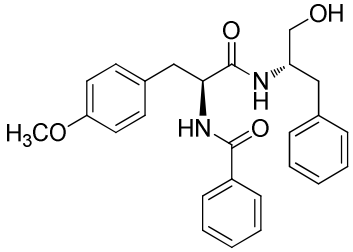
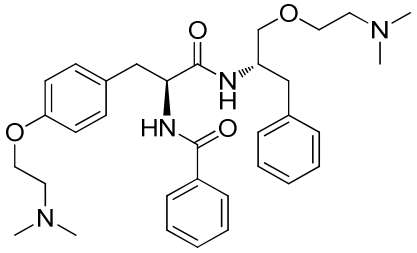
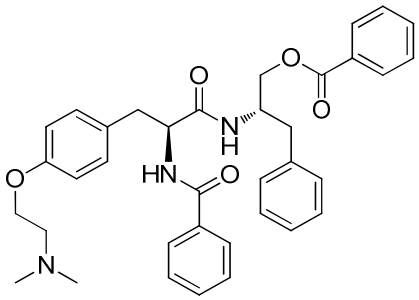
[a]Reaction conditions: (a) 0.75 M NaOH, 2 °C, 1 h, 84% yield; (b) IBCF, NMM, DMF, CH_2Cl_2, -5 °C, 1 h, 78% yield; (c) 2.0 M NaOH, DMF, 30 °C, 4 h, 79% yield; (d) K_2CO_3, 1,4-dioxane, 90 °C, 2 h, 89% yield.

RESULTS AND DISCUSSION

A combination of liquid chromatography and mass spectrometry (LC-MS) was applied to separate and identify the relative impurities in **Y101**. Six impurities (Table 1) were verified and their structures were confirmed by NMR, MS and IR.

Table 1. Structures of impurities A-F

Entry	Name	Structure	Relative Retention Time [a]

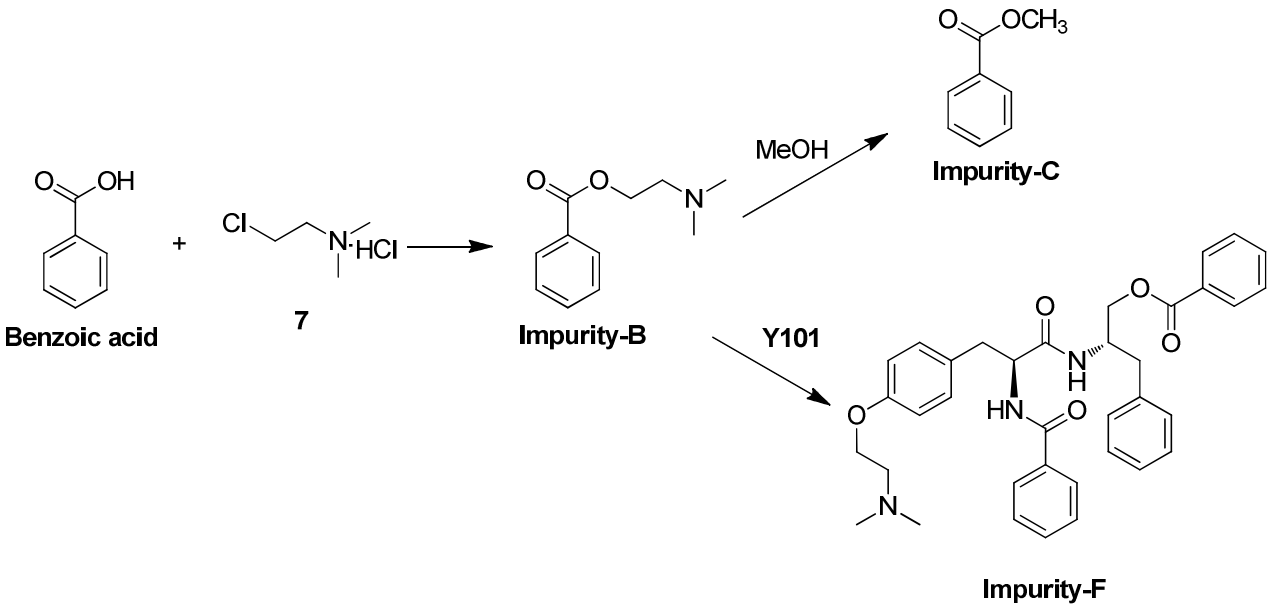
1	Impurity-**A**		0.56
2	Impurity-**B**		0.68
3	Impurity-**C**		0.71
4	Impurity-**D**		0.95
5	Impurity-**E**		2.59
6	Impurity-**F**		5.03

[a]Agilent 1100 HPLC; column: phenomenex Gemini-NX C18 (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: 0.8 mL/min; injection volume: 10 μL; wavelength for UV detection: 226 nm.

Impurity A. Impurity **A** was identified as a starting material of **Y101** and was observed in a range of 0.12-0.27%. Due to structural similarity to **Y101**, the purification process in ethyl acetate and other solvents could not remove impurity **A** effectively.

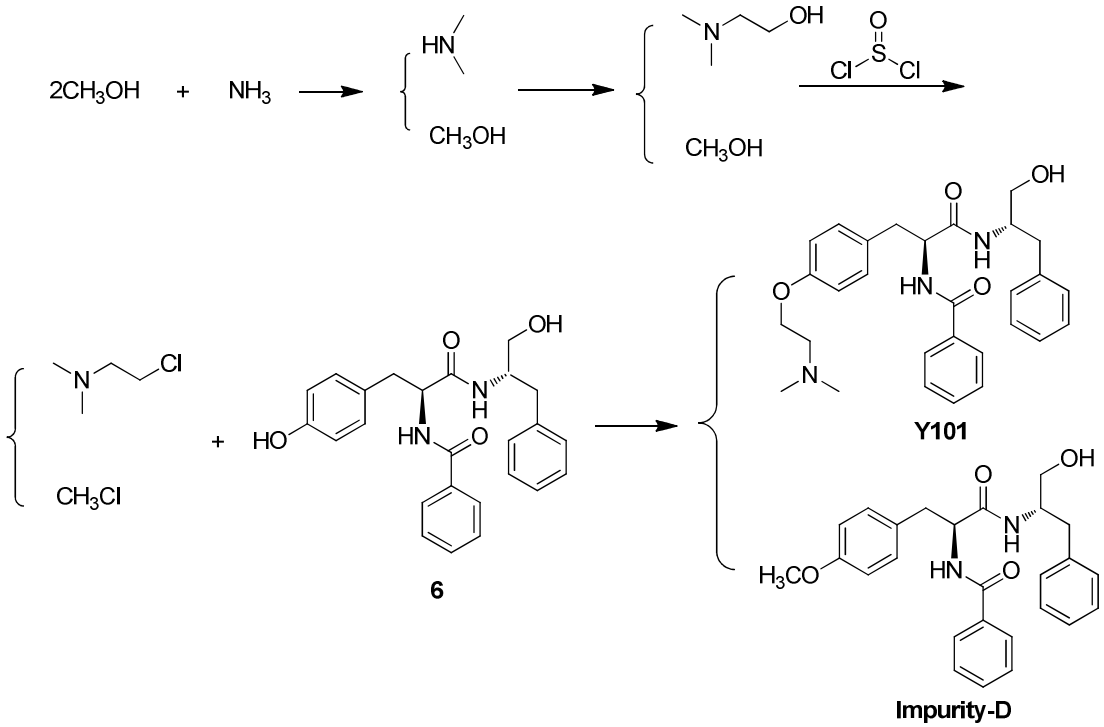
Impurities B, C and F. Impurities **B**, **C** and **F** were observed in the range of 0.16-0.49% in the laboratory experimental studies. Impurity **B** might be generated due to the presence of trace benzoic acid during the reaction of **6** to **Y101**. The synthetic pathway for impurity **B** was shown in Scheme 2. Impurity **C** was likely introduced by reaction of impurity **B** with MeOH which was used for purification in the final step (Scheme 2). Similarly, impurity **F** might be formatted by reaction of **Y101** with small amounts of impurity **B** (Scheme 2).

Scheme 2. Formation of impurities B, C and F



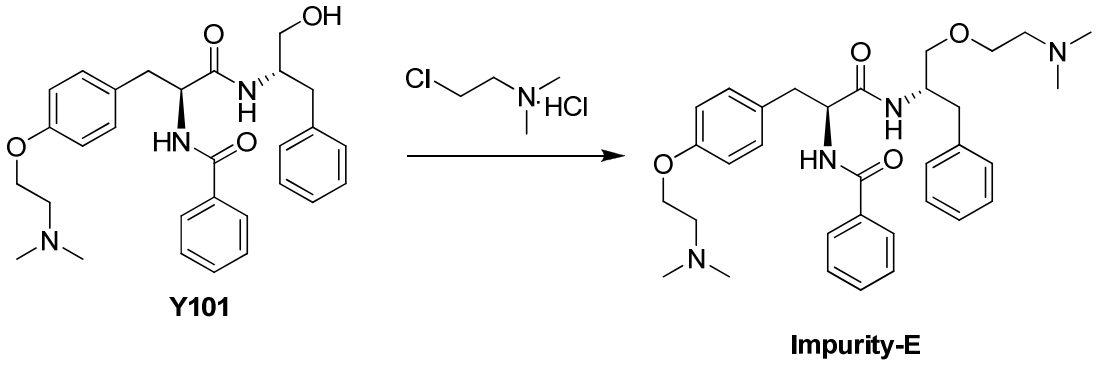
Impurity D. Impurity **D** was isolated from the filtrate of **Y101** recrystallization**,** the structure was confirmed by NMR and MS. This impurity was probably formed due to small amounts of CH_3Cl presented in compound **7** (Scheme 3).[4a-b]

Scheme 3. Formation of impurity D



Impurity E. The reaction of **Y101** with **7** in the presence of potassium hydroxide in 1,4-dioxane might afford impurity **E** (Scheme 4).

Scheme 4. Formation of impurity E



Chiral Control. The most important and critical aspect of **Y101** synthesis was chiral control, which suffered from certain disadvantages such as epimerization, potential high yield loss, number of purifications involved and additional time and resources required for process

development. Furthermore, there was a possibility of forming some stereoisomers, such as stereoisomers **Y101-A**, **Y101-B** and **Y101-C** (Figure 2).

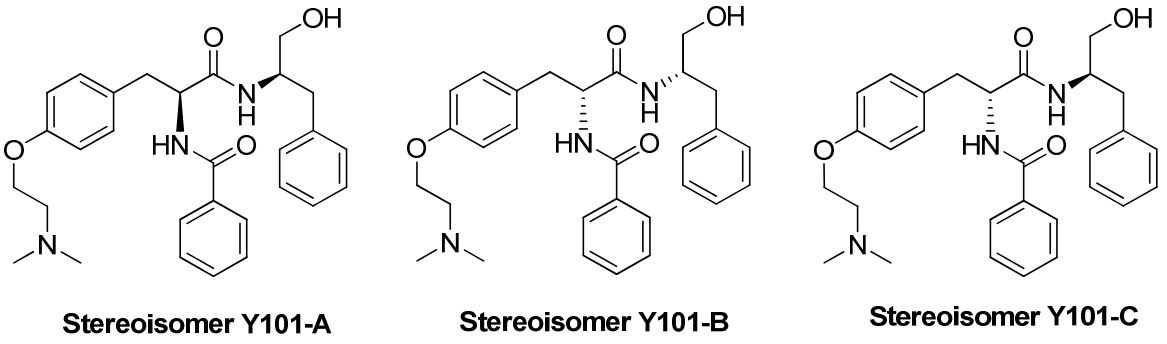


Figure 2. Structures of the stereoisomers of **Y101**.

Stereoisomers **Y101-A**, **Y101-B** and **Y101-C** were potential impurities in the drug substance, since these impurities were chemically distinct and often pharmacologically different from the API, they should be investigated and limited as drug substance impurities. No other relevant references disclosed **Y101** with stereoisomers at a level of less than 0.10%, which was an essential criterion of the bulk drug substance. Hence, the identity and enantiomeric purity of chiral starting materials and chiral reagents should be established. In-process testing for identity and purity should be enantioselective for key intermediates in which additional chiral center(s) have been introduced. [5a-c]

When compound **5** was synthesized by the route depicted in Scheme 1, peptide bond formation was promoted by isobutyl chloroformate (IBCF)-mediated mixed anhydride coupling reactions. IBCF is a cheap and readily available bulk reagent used for quickly activating acids together with *N*-methylmorpholine (NMM) as base at low temperature. Following activation, the peptide coupling reaction is also fast.[6a-f] Yields of the coupling product in the range of 85-90% could be easily achieved. However, in the chiral HPLC profile of compound **5**, small amounts of stereoisomer **5-C** (0.22-0.63 area percent (AP)) was observed (Figure 3).

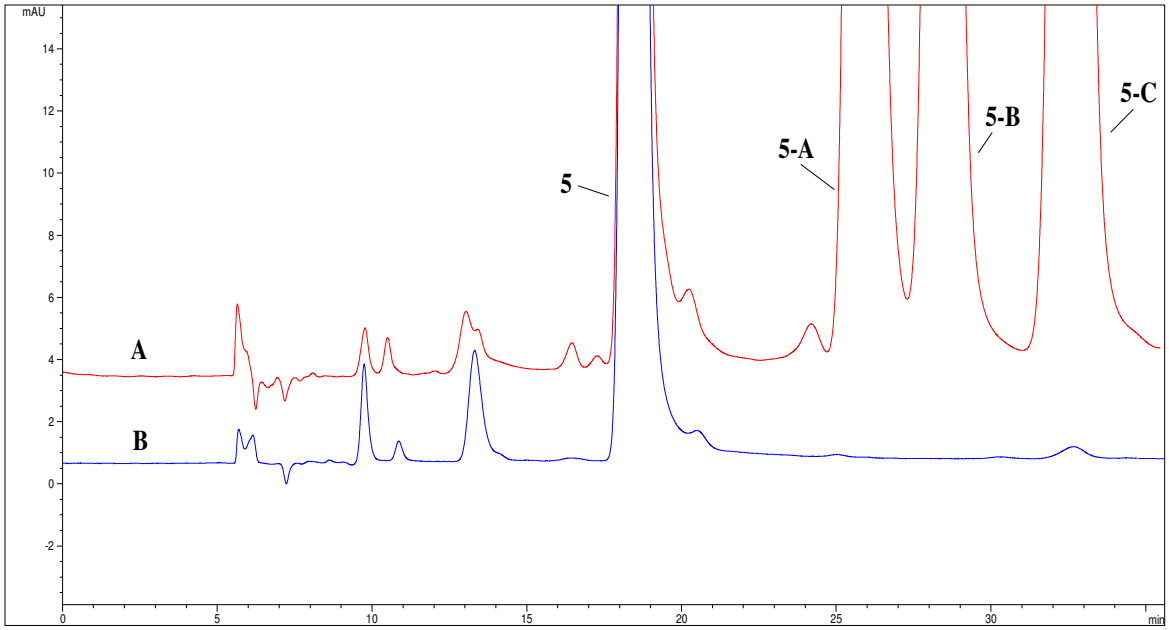
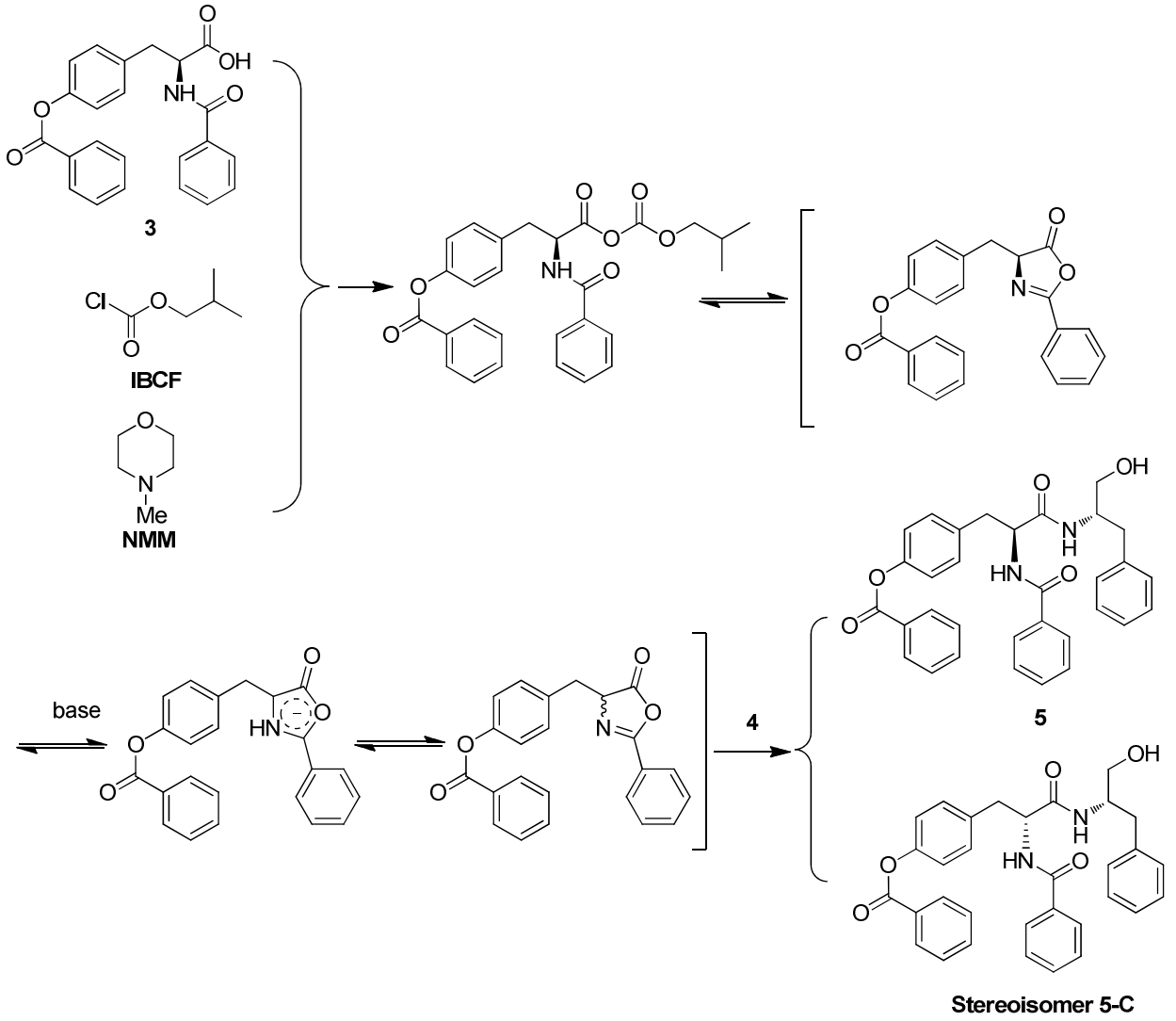


Figure 3. Expanded chiral HPLC chromatograms of intermediate **5**. A: a mixture of intermediate **5**, stereoisomer **5-A** (2S, 2′R), stereoisomer **5-B** (2R, 2′R) and stereoisomer **5-C** (2R, 2′S); B: a crude reaction mixture of intermediate **5**.

Racemization can occur at the C-terminal amino acid residue in the course of a coupling reaction due to the ionization of the a-hydrogen and the formation of an oxazolone intermediate (Scheme 5).[7a-b] Subsequent purification of intermediate **5** using MeOH was effected to afford a single enantiomer.

Scheme 5. Formation of stereoisomer 5-C



In addition, the optimization of various reaction parameters involved in the final step and monitoring the optical purity of reaction products resulted in a dramatic improvement in the optical purity and yield of **Y101** (Table 2).

Table 2. Optimization of reaction condition in the final step

Entry	Base	Mole ratio of base	T(°C)[a]	t_R(h)[b]	Yield (%)	Purity by Chiral HPLC[c]			
						Y101 (%)	Stereoisomer-**A** (%)	Stereoisomer-**B** (%)	Stereoisomer-**C** (%)
1	KOH	2.4	reflux	1	56	46.94	-	52.16	-
2	KOH	2.4	95	2	62	65.63	-	29.78	-

3	KOH	2.4	65	5.5	57	93.34	-	5.60	-
4	K_2CO_3	4	reflux	5	60	99.01	-	-	-
5	K_2CO_3	6	reflux	1.5	80	99.06	-	-	-
6	K_2CO_3	8	reflux	1.5	82	98.95	-	-	-
7	K_2CO_3	6	90	1.5	80	99.25	-	-	-
8	K_2CO_3	6	80	2.5	74	98.92	-	-	-

[a] Temperature of reaction mass.

[b] The reaction was run in 1,4-dioxane with **7** (1.0 eq).

[c] Column: Daicel Chiralcel OD-H (250 mm×4.6 mm, 5 μm); column temperature: 30 °C; mobile phase: n-hexane-ethanol (90:10) containing 0.2% triethylamine; flow rate: 0.8 mL/min; injection volume: 10 μL; wavelength for UV detection: 226 nm.

Various parameters such as mole equivalents of **7** and potassium carbonate, reaction temperature, work-up conditions, and purification process were studied thoroughly. Finally, an efficient and eco-friendly process was developed by improving the original process for the preparation of **Y101**, which was free from stereoisomers, as shown in Figure 4.

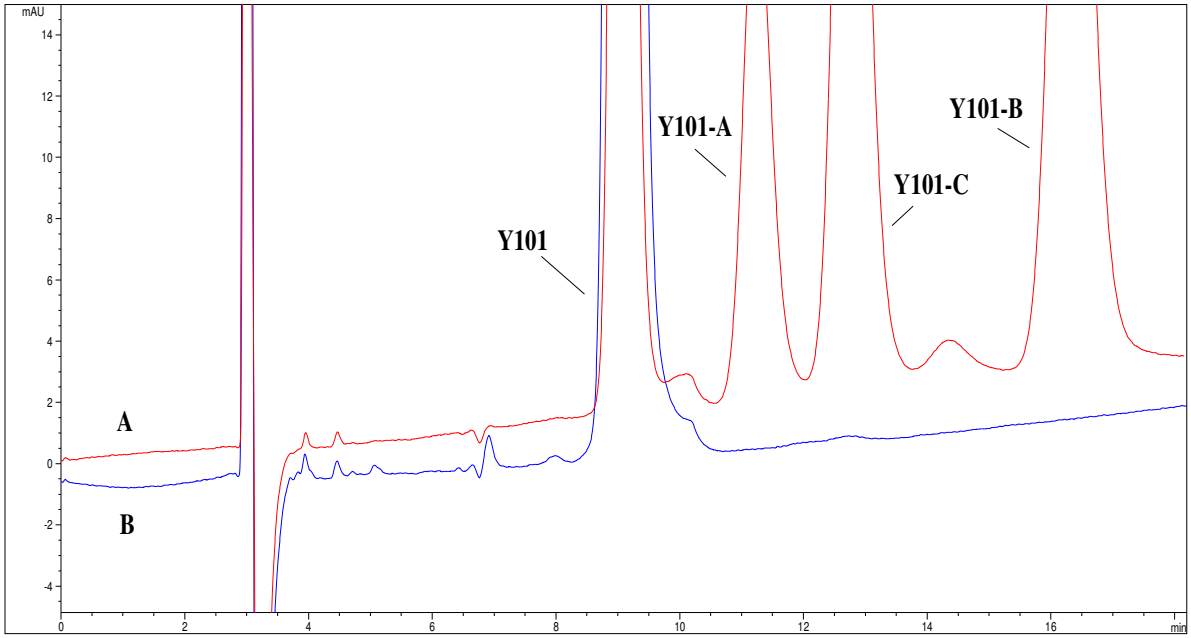
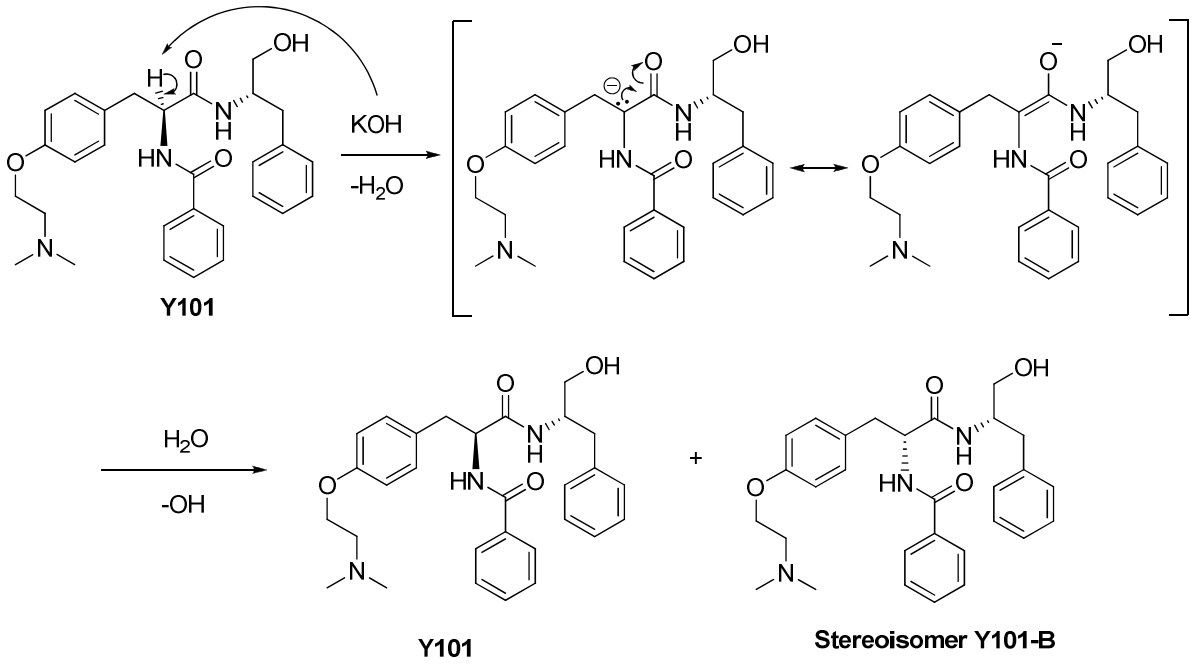


Figure 4. Expanded chiral HPLC chromatograms of **Y101**. A: a mixture of **Y101**, Stereoisomer **Y101-A** (2S, 2′R), Stereoisomer **Y101-B** (2R, 2′S) and Stereoisomer **Y101-C** (2R, 2′R); B: a crude reaction mixture of **Y101**.

The above study indicated that when product was obtained under conditions of entries 1 and 2 in Table 2, stereoisomer **Y101-B** could be detected by chiral HPLC. The mechanism for the formation of stereoisomer **Y101-B** was showed in Scheme 6.[8a-b]

Scheme 6. Formation of stereoisomer Y101-B

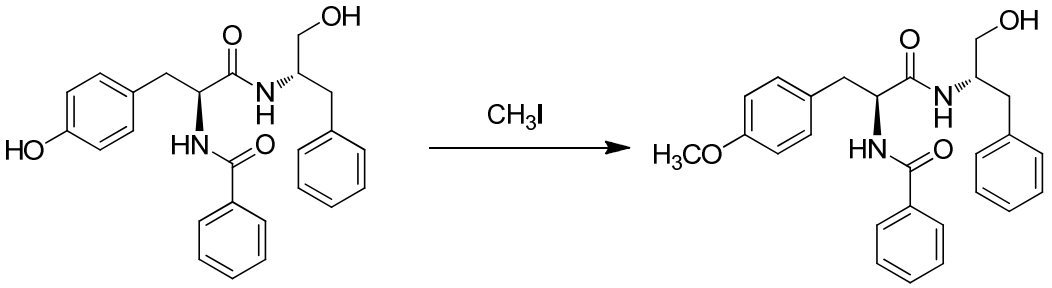


Impurity Synthesis. The quantity required for these impurities to confirm their structure, validation, and use as an analytical standard was significant. Thus, a synthetic route was required for each impurity which could be used to cross-validate the impurity versus the postulated structure and against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

On the basis of Scheme 2, benzoic acid and **7** were identified as starting materials. Impurity **B** was synthesized in the presence of potassium carbonate in 1,4-dioxane. In order to confirm above hypothesis (Scheme 2), the impurity **C** was synthesized by treatment of impurity **B** with MeOH.

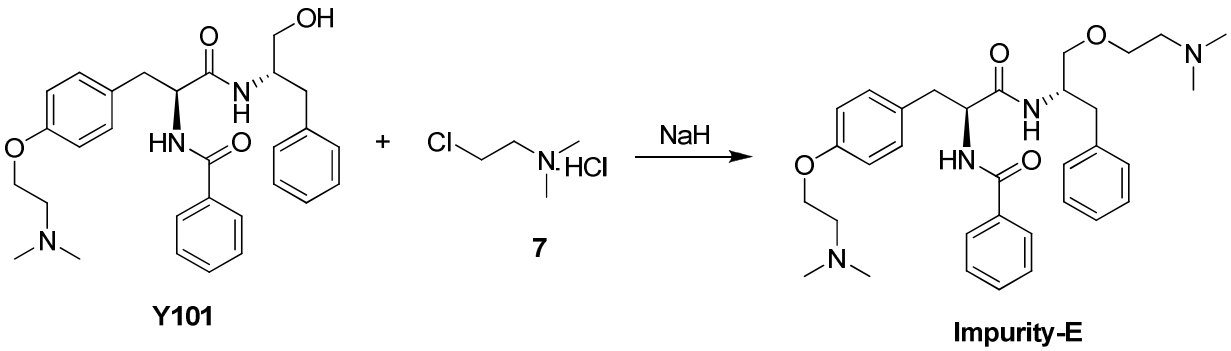
A synthetic pathway for impurities **D** was shown in Scheme 7. Compound **6** and CH_3I reacted in the presence of potassium carbonate in dry DMF at 35 °C.

Scheme 7. Synthesis of impurity D



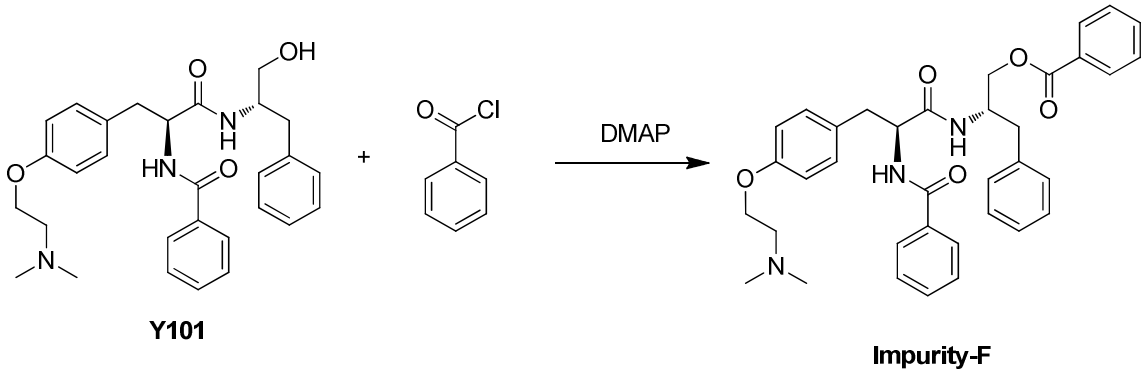
Direct synthesis of impurities **E** and **F** from **Y101** was considered reasonable. As Scheme 8 showed, impurity **E** was obtained by reaction of **Y101** with **7** in the presence of sodium hydride in anhydrous DMF at 0 °C.

Scheme 8. Synthesis of impurity E



Impurity **F** was obtained by reaction of **Y101** with benzoyl chloride in the presence of DMAP (Scheme 9).

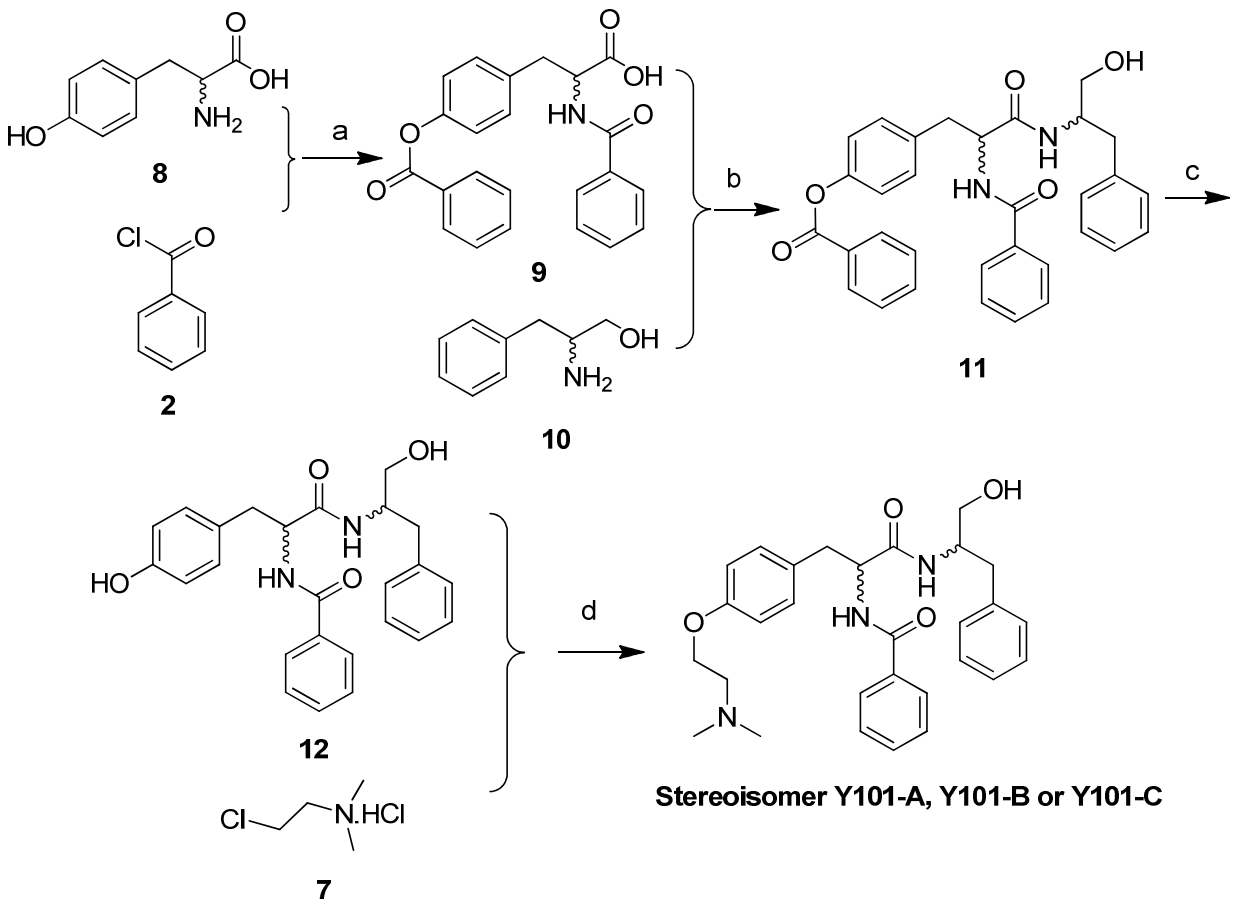
Scheme 9. Synthesis of impurity F



Y101 **Impurity-F**

The synthetic procedures for the preparation of stereoisomers **Y101-A**, **Y101-B** and **Y101-C** were illustrated in Scheme 10. L-tyrosine or D-tyrosine was reacted with **2** to give compound **9**. Compound **9** was reacted with L-phenylalaninol or D-phenylalaninol in the presence of IBCF and NMM to give compound **11**, which was then hydrolyzed in NaOH (2.0 M) to give compound **12**. Reaction of **12** with **7** in the presence of potassium carbonate afforded target stereoisomers **Y101-A**, **Y101-B** and **Y101-C**.

Scheme 10. Synthesis of stereoisomers Y101-A, Y101-B and Y101-C [a]



[a]Reaction conditions: (a) 0.75 M NaOH, 2 °C, 1 h; (b) IBCF, NMM, DMF, CH_2Cl_2, -5 °C, 1 h; (c) 2.0 M NaOH, DMF, 30 °C, 4 h; (d) K_2CO_3, 1,4-dioxane, 90 °C, 2 h.

Impurity Control. According to the synthetic route depicted in Scheme 1, intermediates **3**, **5** and **6** were synthesized in the preparation of **Y101** from L-tyrosine as the starting material. Some impurities in the intermediates **3**, **5** and **6** had severe effects on the quality of **Y101**. Therefore, understanding the relative substances in the intermediates **3**, **5** and **6** were urgently necessary to provide valuable information for quality control.

Based on the production process (Scheme 1), intermediate **3** was synthesized from benzoyl chloride and L-tyrosine through the acylation reaction. Consequently, the presence of benzoic acid (solvolysis reactions of benzoyl chloride) and the incomplete acylation product (compound **13**) in the product was possible (Figure 5). Quantitative results revealed that **13** was major

impurity (2.2-3.2%) in the crude reaction mixture, while benzoic acid was observed in the range of 0.28-0.49%. These two impurities that contained in intermediate **3** showed little impact on the next step, so there was no purification involved.

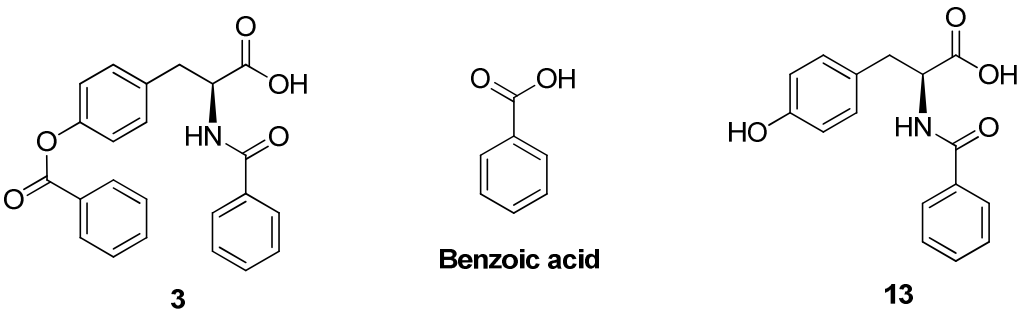


Figure 5. Structures of intermediate **3** and the impurities.

Intermediate **5** was synthesized through IBCF and NMM with intermediate **3** and compound **4** as the starting materials. Therefore, the residue of **3** and **4** in the product was likely. In addition, impurities originating in intermediate **3** could be carried into the reaction, and could also be transformed by the process into new impurities (Figure 6). Compared with other potential impurities, impurity **14** (0.36-0.58%), Stereoisomer **5-C** (0.22-0.63%) and intermediate **3** (0.17-0.45%) were the greatest concern because their residue would greatly affect the quality of intermediate **6**. Therefore, MeOH was finally chosen for the purification of intermediate **5**.

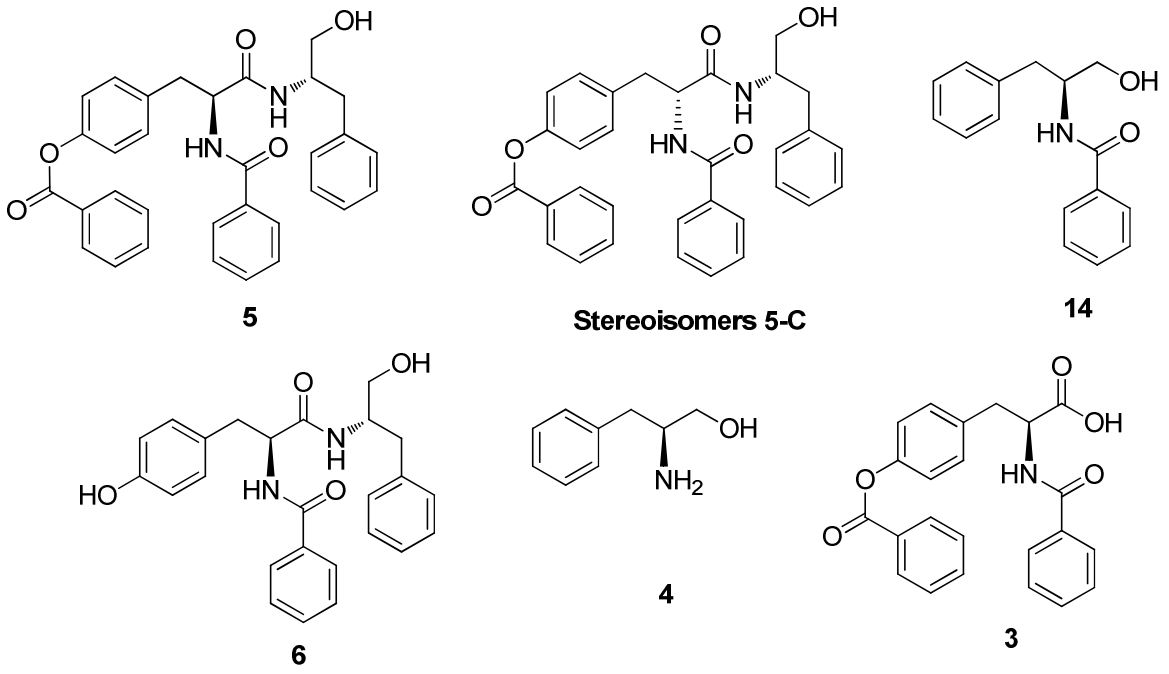


Figure 6. Structures of intermediate **5** and the impurities.

Intermediate **6** was synthesized by hydrolyzation of intermediate **5** in NaOH (2.0 M). Benzoic acid was found as major impurity in the product and observed in the range of 2.3-3.6% in the lab experimental studies (Figure 7).

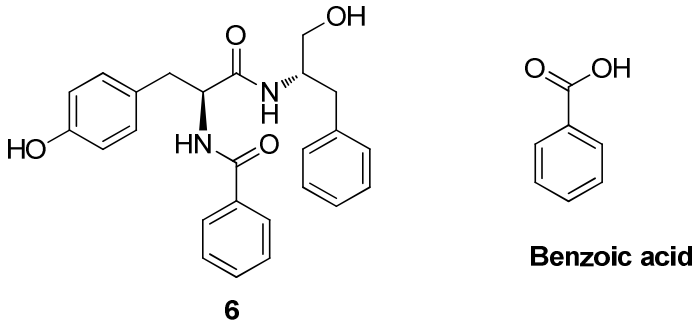
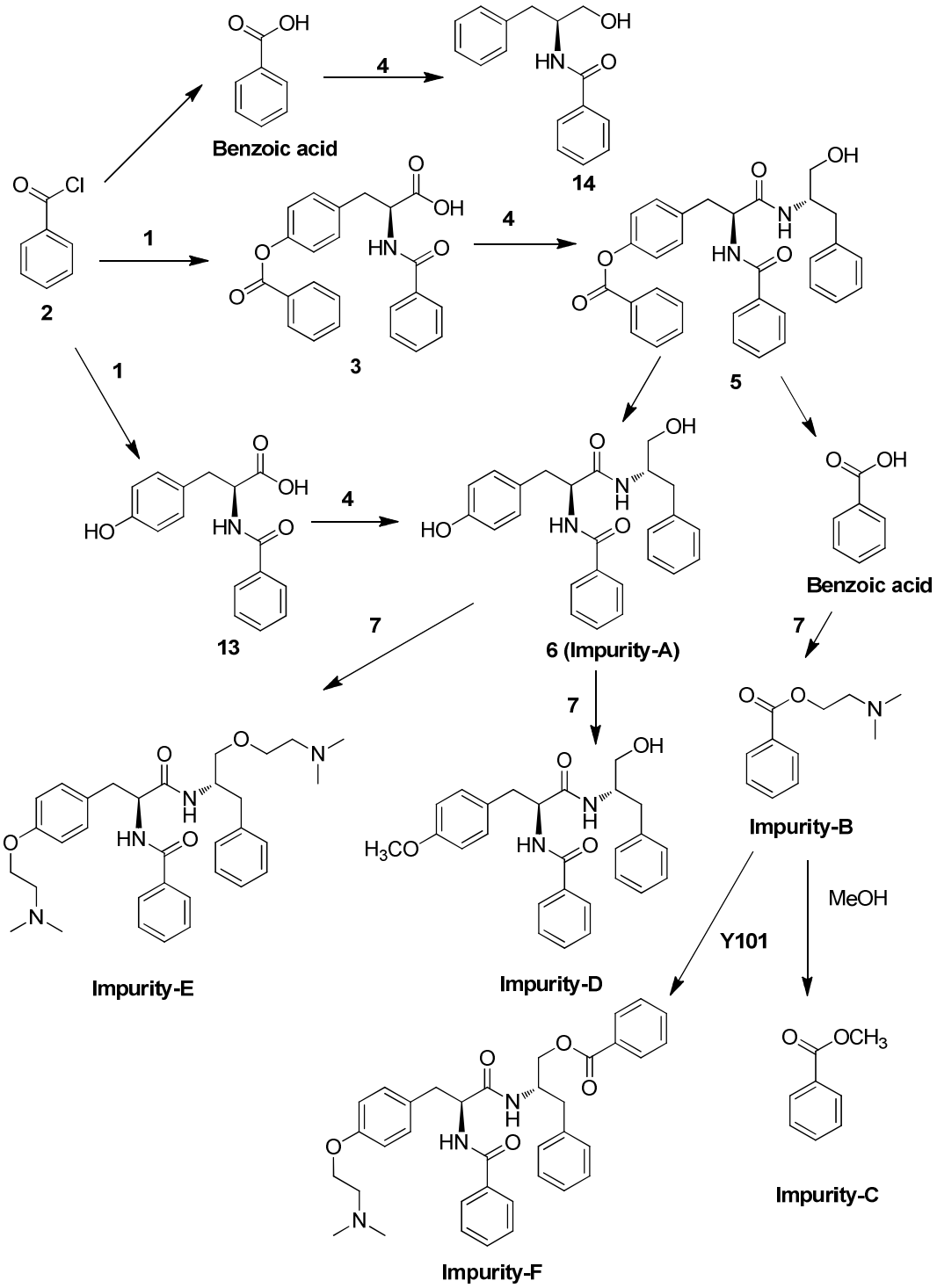


Figure 7. Structures of intermediate **6** and the impurities.

Based on the initial input on potential impurities by the process research, impurity tracking from starting materials, through intermediates and finally to API manufacturing process was

carried out (Scheme 11). The potential impurities of each stage postulated during the process research were used as the basis for quality control.

Scheme 11. Impurity tracking of Y101 process



Following Scheme 11, impurities **B**, **C** and **F** might be formed due to the presence of benzoic acid during the reaction of **6** to **Y101**. The percent of benzoic acid in compound **6** was an important aspect to reduce the formation of impurities **B**, **C** and **F**. In order to identifying the suitable solvents for purification, ethyl acetate was finally chosen for the purification of compound **6**, and the content of benzoic acid in compound **6** was reduced from 2.81% to 0.15%. When **Y101** was obtained by reaction of the purified **6** with **7**, impurities **B**, **C** and **F** were reduced to lower than 0.1%.

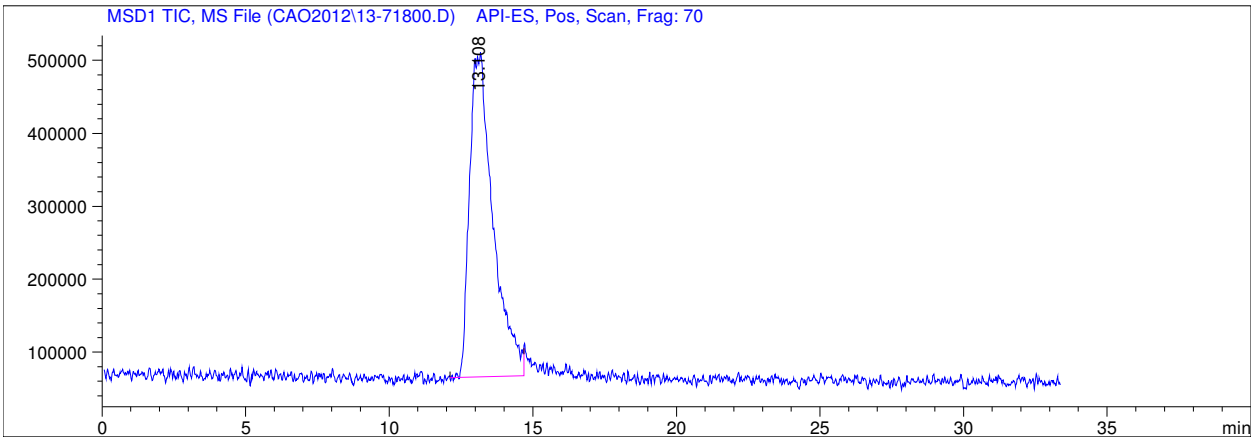
Impurity **A** (compound **6**) was the most critical impurity and it could be controlled to the level 0.1% during the final step. This was achieved by controlling the mole ratios of **7** and potassium carbonate, in such a way that nearly all compound **6** was converted to **Y101**.

Due to different physicochemical properties with **Y101**, impurity **D** was controlled by purification under different pH. Thus, the reaction mixture of **6** was poured into water and extracted twice with ethyl acetate, the combined organic phase was abstracted with 0.1 M HCl (1.2 eq), the aqueous layer was adjust to pH 8-9 using 1.0 M NaOH, and then extracted with ethyl acetate, the organic phase was washed with brine, and then dried over $MgSO_4$. Filtration and solvent evaporation gave purified **Y101**. Additional, this improved approach was implemented minimizing impurities **A** and **C** to the level accepted by ICH.

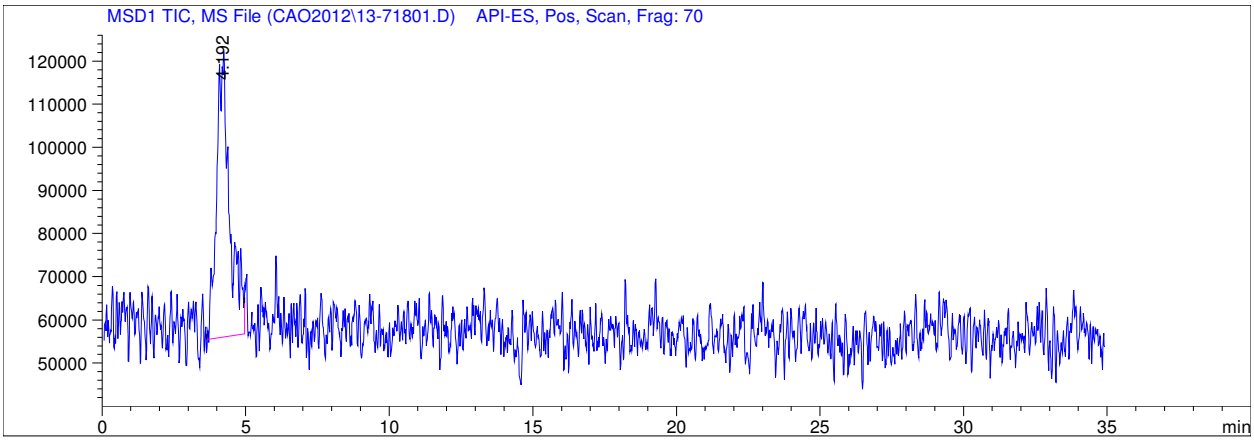
Impurity **E** was a process impurity. If base was changed from potassium hydroxide to potassium carbonate in the final step, impurity **E** disappeared in **Y101**.

As starting material of **Y101**, alkylating agents like compound **7** immediately attracted our attention as potential genotoxic impurity. In view of patient safety, compound **7** need to be carefully controlled as potential impurities in the drug substance. The process for purifying removal of compound **7** from API was achieved by recrystallization from MeOH- Ether. LC-MS

was validated for the assessment of compound **7** in API, with method detection limits in the 0.1 μg/g range. The analysis results showed that this purification process ensures **Y101** free from compound **7** (Figure 8).



A



B

Figure 8. Mass spectra of **Y101**(A) and compound **7** (B).

CONCLUSION

The structures of impurities **A-F,** stereoisomers **Y101-(A-C)** and their origins of formation during the preparation of **Y101** were identified. The structural knowledge led to the development of a rework protocol that purged out these impurities to the level accepted by ICH.

EXPERIMENTAL SECTION

All materials were purchased from commercial suppliers. Unless specified otherwise, all reagents and solvents were used as supplied by manufacturers. FT IR spectra were recorded on the solid state as KBr dispersion using BRUKER-VECTOR22 FT IR spectrophotometer. Melting points were determined with the X-4 melting point apparatus. The ^{1}H NMR and ^{13}C NMR spectra were measured in DMSO-d_6 on INOVA 400 MHz FT NMR spectrometer. Chemical shifts are reported in δ (ppm) relative to TMS (δ 0.0), DMSO-d_6 (δ 39.50). Mass spectra were determined on HP 1100 LC-MSD mass spectrometer.

Synthesis of Impurity A. To a solution of **1** (7.25 g, 0.04 mol) and NaOH (4.9 g, 0.12 mol) in water (160 mL) was added **2** (11.3 g, 0.08 mol) dropwise at 2-4 °C. The mixture was stirred for 1 h , then adjusted to pH 2-3 with concentrated hydrochloric acid. The reaction mixture was filtered, the resulting filter cake was dried and recrystallized from ethyl acetate to afford target compound **3** (13.1 g, 98.78% purity) in 84% yield.

Mp: 214-215 °C; $[\alpha]_D^{23}$ -79 (*c* 0.5, DMF); IR (KBr, cm^{-1}) 3287, 1735, 1640, 1538, 1267, 1198, 1062, 707; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 12.84 (s, 1H), 8.77 (d, *J* = 8.0 Hz, 1H), 8.11 (d, *J* = 7.6 Hz, 2H), 7.82 (d, *J* = 7.6 Hz, 2H), 7.73 (t, *J* = 7.6 Hz, 1H), 7.59 (t, *J* = 7.2 Hz, 2H), 7.53 (t, *J* = 7.2 Hz, 1H), 7.46 (t, *J* = 7.2 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.20 (d, *J* = 8.4 Hz, 2H), 4.70-4.64 (m, 1H), 3.28-3.10 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 173.2, 166.5, 164.6, 149.1, 136.0, 134.0, 133.9, 131.4, 130.2, 129.8, 129.0, 128.3, 127.4, 121.6, 54.2, 35.6; HRMS (ESI): Calcd for $C_{23}H_{18}NO_5$ [M-H]$^-$ 388.1185, Found 388.1189.

IBCF (4.51 g, 0.033 mol) was added dropwise to the mixture of **3** (11.7 g, 0.03 mol), **4** (5.0 g, 0.033 mol), NMM (4.55 g, 0.045 mol) and DMF (50 mL) in CH_2Cl_2 (200 mL) at -5 °C within 30 min. The mixture was stirred for 30 min 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, dried with $MgSO_4$. Filtration and solvent evaporation gave a residue which was recrystallized from MeOH to afford target compound **5** (12.2 g, 99.55% purity) in 78% yield.

Mp: 240-241 °C; $[\alpha]_D^{23}$ -82 (*c* 0.5, DMF); IR (KBr, cm^{-1}) 3310, 1730, 1633, 1542, 1276, 1197, 1066, 1047, 713; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.56 (d, *J* = 8.4 Hz, 1H), 8.10 (d, *J* = 7.6 Hz, 2H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.83 (d, *J* = 7.6 Hz, 2H), 7.73 (t, *J* = 7.6 Hz, 1H), 7.59 (d, *J* = 7.6 Hz, 2H), 7.53 (t, *J* = 7.2 Hz, 1H), 7.46 (t, *J* = 7.2 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 7.2 Hz, 2H), 7.19 (d, *J* = 7.2 Hz, 2H), 7.17 (d, *J* = 8.4 Hz, 2H), 7.13 (t, *J* = 7.2 Hz, 1H), 4.85 (t, *J* = 5.2 Hz, 1H), 4.77-4.71 (m, 1H), 3.91-3.97 (m, 1H), 3.41-3.30 (m, 2H), 3.11-2.67 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 170.9, 166.2, 164.6, 149.0, 140.0, 136.2, 134.0, 134.0, 131.3, 130.2, 129.7, 129.2, 129.0, 128.2, 128.1, 127.4, 125.9, 121.4, 62.2, 54.8, 52.5, 36.6, 36.5; HRMS (ESI): Calcd for $C_{32}H_{30}N_2O_5Na$ $[M+Na]^+$ 545.2052, Found 545.2057.

NaOH (2 M, 30 mL) was added to the solution of compound **5** (10.44 g, 0.02 mol) in DMF (35 mL). After stirring at 30 °C for 4 h, the mixture was acidified to pH 2-3 with concentrated hydrochloric acid, and partitioned between ethyl acetate and water. The organic phase was separated and sequentially washed with saturated $NaHCO_3$ solution and brine, dried over $MgSO_4$ and evaporated in vacuo to give the impurity **A** (6.6 g, 99.52% purity) in 79% yield.

Mp: 213-214 °C; $[\alpha]_D^{23}$ -68 (*c* 1.0, MeOH); IR (KBr, cm^{-1}) 3315, 1632, 1518, 1250, 1078, 1037, 694; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 9.17 (s, 1H), 8.46 (d, *J* = 8.8 Hz, 1H), 7.90 (d, *J* = 8.4 Hz, 1H), 7.81 (d, *J* = 6.8 Hz, 2H), 7.52 (t, *J* = 7.2 Hz, 1H), 7.45 (t, *J* = 7.2 Hz, 2H), 7.24-7.10 (m, 7H), 6.63 (d, *J* = 8.4 Hz, 2H), 4.84 (t, *J* = 5.2 Hz, 1H), 4.64-4.58 (m, 1H), 3.94-3.90 (m, 1H), 3.38-3.26 (m, 2H), 2.96-2.65 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 171.1, 166.1,

155.8, 139.1, 134.2, 131.3, 130.1, 129.2, 128.4, 128.2, 128.1, 127.5, 125.9, 114.9, 62.2, 55.2, 52.5, 36.6, 36.5; HRMS (ESI): Calcd for $C_{25}H_{26}N_2O_4Na$ $[M+Na]^+$ 441.1790, Found 441.1795.

Synthesis of Impurity B. To a solution of **7** (1.0 g, 6.7 mmol) in 1,4-dioxane (35 mL) were added benzoic acid (1.22 g, 10 mmol) and potassium carbonate (5.53 g, 40 mmol), the reaction mixture was stirred at 90 °C for 1.5 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 buff oily compound. The compound was purified using column chromatography (silica gel, CH_2Cl_2/ triethylamine, 200/1) to give impurity **B** as oily compound (1.1 g, 99.31% purity) in 85% yield.

IR (KBr, cm^{-1}) 3418, 2950, 1644, 1054, 1032, 1016, 673; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 7.95 (d, J = 7.2 Hz, 2H), 7.64 (t, J = 7.6 Hz, 1H), 7.52 (t, J = 7.6 Hz, 2H), 4.34 (t, J = 5.6 Hz, 2H), 2.60 (t, J = 5.6 Hz, 2H), 2.20 (s, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 165.7, 133.3, 129.8, 129.1, 128.8, 62.5, 57.2, 54.3; MS (ESI): 216.1$[M+Na]^+$.

Synthesis of Impurity C. Impurity **B** (0.97 g, 5 mmol) was dissolved in 25 mL of MeOH and the reaction mixture was stirred at 50 °C for 24 h. The reaction mixture was concentrated under reduced pressure gave buff oily compound, the compound was purified using column chromatography (silica gel, cyclohexane/EtOAC, 50/1) to give impurity **C** as oily compound (0.6 g, 99.26% purity) in 88% yield.

IR (KBr, cm^{-1}) 3417, 2949, 1648, 1052, 1032, 1021, 718; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 7.98 (d, J = 8.8 Hz, 2H), 7.67 (t, J = 7.6 Hz, 1H), 7.54 (t, J = 8.0 Hz, 2H), 3.86 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 166.2, 133.3, 129.6, 129.1, 128.8, 52.1; MS (ESI): 159.1 $[M+Na]^+$.

Synthesis of Impurity D. To a suspension of compound **6** (2.1 g, 5 mmol) in dry DMF (25 mL) were added CH_3I (1.7 g, 12 mmol) and potassium carbonate (2.76 g, 20 mmol), the reaction mixture was stirred at 35 °C for 2.5 h. Ethyl acetate and water were charged, the mixture was stirred for 30 min. The organic layer was washed with brine, and then dried over $MgSO_4$. Filtration and solvent evaporation gave yellow solid, the solid purified using column chromatography (silica gel, $CHCl_3$/MeOH, 60/1) to give impurity **D** (1.79 g, 99.63% purity) in 83% yield.

Mp: 202-204 °C; $[\alpha]_D^{25}$ -68 (*c* 0.91, MeOH); IR (KBr, cm^{-1}) 3293, 1634, 1539, 1303, 1248, 1179, 1034, 696; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.42 (d, *J* = 8.4 Hz, 1H), 7.84 (d, *J* = 8.4 Hz, 1H), 7.78 (d, *J* = 7.2 Hz, 2H), 7.51 (t, *J* = 7.2 Hz, 1H), 7.44 (t, *J* = 7.2 Hz, 2H), 7.21-7.11 (m, 7H), 6.79 (d, *J* = 8.4 Hz, 2H), 4.79 (t, *J* = 5.2 Hz, 1H), 4.62-4.617 (m, 1H), 3.89-3.87 (m, 1H), 3.67 (s, 3H), 3.34-3.241 (m, 2H), 2.96-2.64 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 171.1, 166.2, 157.8, 139.1, 134.1, 131.4, 130.3, 129.3, 128.3, 128.2, 127.5, 126.0, 113.5, 62.3, 55.2, 55.0, 52.5, 36.5; MS (ESI): 455.1 $[M+Na]^+$.

Synthesis of Impurity E. NaH (0.4 g, 10 mmol) was dissolved in 20 mL of anhydrous DMF and cooled to 0 °C under argon atmosphere. **Y101** (0.49 g, 1 mmol) was added. After 30 min, **7** (0.72 g, 5 mmol) was added as a solid, and the reaction mixture was stirred at 25 °C for 24 h. Small amount of ethanol was charged, the mixture was stirred for 30 min, then the reaction mixture was extracted with ethyl acetate, the extract was washed with water, and brine. The organic layer was dried ($MgSO_4$), filtered and concentrated in vacuo to give yellow solid , the solid was purified using column chromatography (silica gel, $CHCl_3$/MeOH/triethylamine, 50/1/0.3)to give impurity **E** (0.45 g, 98.53% purity) in 80% yield.

Mp: 160-162 °C; $[\alpha]_D^{25}$ -42 (*c* 1.04, MeOH); IR (KBr, cm^{-1}) 3299, 2942, 2859, 2767, 1632, 1533, 1454, 1388, 1307, 1246, 1129, 1042, 695; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.46 (d, *J* = 8.4 Hz, 1H), 7.98 (d, *J* = 8.4 Hz, 1H), 7.80 (d, *J* = 7.2 Hz, 2H), 7.521 (t, *J* = 7.2 Hz, 1H), 7.45 (t, *J* = 7.2 Hz, 2H), 7.221-7.14 (m, 7H), 6.80 (d, *J* = 8.4 Hz, 2H), 4.65-4.59 (m, 1H), 4.05-4.02 (m, 1H), 3.97 (t, *J* = 6.4 Hz, 2H), 3.45 (t, *J* = 6.4 Hz, 2H), 3.28 (d, *J* = 5.2 Hz, 2H), 2.96-2.68 (m, 4H), 2.58 (t, *J* = 6.4 Hz, 2H), 2.42 (t, *J* = 6.4 Hz, 2H), 2.19 (s, 6H), 2.17 (s, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 171.1, 166.1, 157.0, 138.6, 134.1, 131.3, 130.2, 129.2, 128.2, 128.1, 127.4, 126.0, 114.0, 71.3, 68.5, 65.5, 58.0, 57.7, 55.2, 50.1, 45.5, 45.4, 36.8, 36.5; MS (ESI): 561.5 $[M+H]^+$.

Synthesis of Impurity F. **Y101** (0.49 g, 1 mmol) was dissolved in 10 mL of anhydrous pyridine under argon atmosphere, followed by addition of benzoyl chloride (0.28 g, 2 mmol) and DMAP (0.1 g, 0.82 mmol). The reaction mixture was stirred at 50 °C for 4 h. The reaction mixture was poured into water, the solid was filtered and purified using column chromatography (silica gel, $CHCl_3$/MeOH/, 40/1) to give impurity **F** (0.53 g, 99.44% purity) in 90% yield.

Mp: 178-180 °C; $[\alpha]_D^{25}$ -20 (*c* 0.99, MeOH); IR (KBr, cm^{-1}) 3284, 1722, 1634, 1544, 1383, 1281, 1245, 1178, 1132, 1027, 708; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.36 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 8.4 Hz, 1H), 7.97 (d, *J* = 7.2 Hz, 2H), 7.78 (d, *J* = 6.8 Hz, 2H), 7.64 (t, *J* = 8.0 Hz, 1H), 7.50 (t, *J* = 7.6 Hz, 3H), 7.42 (t, *J* = 7.6 Hz, 2H), 7.27-7.135 (m, 7H), 6.73 (d, *J* = 8.8 Hz, 2H), 4.68-4.62 (m, 1H), 4.36-4.33 (m, 1H), 4.26-4.22 (m, 1H), 4.16-4.12 (m, 1H), 3.91 (t, *J* = 6.0 Hz, 2H), 2.90-2.83 (m, 4H), 2.53 (t, *J* = 6.4 Hz, 2H), 2.19 (s, 6H,); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.4, 166.1, 165.7, 156.9, 134.1, 133.4, 132.3, 131.3, 130.1, 129.6, 129.4, 129.3, 129.2, 128.7, 128.4, 128.3, 128.2, 127.5, 126.3, 114.0, 65.5, 65.3, 57.5, 55.2, 49.2, 45.3, 36.7, 36.5; MS (ESI): 594.2 $[M+H]^+$.

Synthesis of Y101. Compound **6** (2.1 g, 5 mmol) was dissolved in 25 mL of 1,4-dioxane under argon atmosphere. **7** (0.9 g, 6 mmol) and potassium carbonate (4.2 g, 30 mmol) were added. The reaction mixture was stirred at 90 °C for 2 h. The reaction mixture was poured into water, the solid was filtered and recrystallized from MeOH- Ether to give **Y101** (2.18 g, 99.53% purity) in 89% yield.

Mp: 177-179 °C; $[\alpha]_D^{26}$ -66 (*c* 1.0, MeOH); IR (KBr, cm^{-1}) 3314, 1634, 1247, 1043, 699; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.46 (d, *J* = 8.4 Hz, 1H), 7.87 (d, *J* = 8.4 Hz, 1H), 7.80 (d, *J* = 7.2 Hz), 7.52 (t, *J* = 7.2 Hz, 1H), 7.45 (t, *J* = 7.2 Hz, 2H), 7.25-7.20 (m, 4H), 7.18 (t, *J* = 7.6 Hz, 2H), 7.12 (t, *J* = 6.8 Hz, 1H), 6.80 (d, *J* = 8.4 Hz, 2H), 4.81 (t, *J* = 5.2 Hz, 1H), 4.62 (m, 1H), 3.96 (t, *J* = 6.09 Hz, 2H), 3.94-3.84 (m, 1H), 3.38-3.24 (m, 2H), 2.99-2.65 (m,4H), 2.56 (t, *J* = 6.0 Hz, 2H), 2.17 (s, 6H); ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.1, 166.1, 160.0, 139.0, 134.1, 131.3, 130.3, 130.2, 129.2, 128.2, 128.1, 127.5, 125.9, 114.0, 65.6, 62.2, 57.8, 55.1, 52.5, 45.6, 36.5; HRMS (ESI): Calcd for $C_{29}H_{36}N_3O_4$ $[M+H]^+$ 490.2706, Found 490.2704.

Synthesis of Stereoisomers Y101-A, Y101-B and Y101-C. According to Scheme 8, L-tyrosine, D-tyrosine, L-phenylalaninol and D-phenylalaninol were identified as starting materials. The synthetic procedures for stereoisomers **A**, **B** and **C** were similar with that of **Y101**.

Stereoisomer Y101-A. Mp: 171-172 °C; $[\alpha]_D^{26}$ +15.4 (*c* 1.04, MeOH); IR (KBr, cm^{-1}) 3420, 2855, 1635, 1383, 1240, 1028, 701; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.40 (d, *J* = 8.4 Hz, 1H), 8.06 (d, *J* = 8.4 Hz, 1H), 7.79 (d, *J* = 7.2 Hz, 2H), 7.50 (t, *J* = 7.2 Hz, 1H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.27-7.13 (m, 6H), 6.79 (d, *J* = 8.8 Hz, 2H), 4.91 (t, *J* = 5.2 Hz, 1H), 4.62-4.60 (m, 1H), 4.01-3.92 (m, 1H), 3.95 (t, *J* = 6.0 Hz, 2H), 3.46-3.35 (m, 2H), 2.93-2.62 (m, 4H), 2.55 (t, *J* = 6.0 Hz, 2H), 2.17 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 171.1, 166.0, 156.9, 139.2,

134.1, 131.3, 130.3, 130.2, 129.3, 128.2, 128.0, 127.4, 126.0, 113.9, 65.6, 63.0, 57.8, 55.2, 52.5, 45.6, 36.8, 36.7; MS (ESI): 490.5 $[M+H]^+$.

Stereoisomer Y101-B. Mp: 169-170 °C; $[\alpha]_D^{26}$ -20.9 (*c* 0.89, MeOH); IR (KBr, cm^{-1}) 3297, 1635, 1538, 1240, 1028, 789, 701; ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.40 (d, *J* = 8.4 Hz, 1H), 8.05 (d, *J* = 8.4 Hz, 1H), 7.79 (d, *J* = 7.2 Hz, 2H), 7.50 (t, *J* = 7.2 Hz, 1H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.28-7.12 (m, 7H), 6.79 (d, *J* = 8.8 Hz, 2H), 4.89 (t, *J* = 5.2 Hz, 1H), 4.61-4.59 (m, 1H), 4.01-3.91 (m, 1H), 3.95 (t, *J* = 6.0 Hz, 2H), 3.45-3.35 (m, 2H), 2.93-2.62 (m, 4H), 2.55 (t, *J* = 6.0 Hz, 2H), 2.17 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 171.1, 167.0, 156.9, 139.2, 134.1, 131.3, 130.3, 130.2, 129.3, 128.2, 128.0, 127.4, 126.0, 113.9, 65.6, 63.0, 57.8, 55.2, 52.5, 45.56, 36.8, 36.7; MS (ESI): 490.5 $[M+H]^+$.

Stereoisomer Y101-C. Mp: 177-178 °C; $[\alpha]_D^{26}$ +64.2 (*c* 1.0, MeOH); IR (KBr, cm^{-1}) 3301, 1658, 1635, 1537, 1246, 1180, 1078, 699; ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.48 (d, *J* = 8.5 Hz, 1H), 7.90 (d, *J* = 8.3 Hz, 1H), 7.81 (d, *J* = 7.2 Hz, 2H), 7.52 (t, *J* = 7.2 Hz, 1H), 7.45 (t, *J* = 7.2 Hz, 2H), 7.25-7.16 (m, 6H), 7.13 (t, *J* = 7.2 Hz, 1H), 6.81 (d, *J* = 8.8 Hz, 2H), 4.84 (s, 1H), 4.67-4.62 (m, 1H), 3.96 (t, *J* = 6.0 Hz, 2H), 3.95-3.87 (m, 1H), 3.40-3.25 (m, 2H), 2.98-2.66 (m, 4H), 2.56 (t, *J* = 5.9 Hz, 2H), 2.18 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 171.1, 166.1, 160.0, 139.0, 134.1, 131.3, 130.3, 130.2, 129.2, 128.2, 128.1, 127.4, 125.9, 114.0, 65.6, 62.2, 57.8, 55.1, 45.6, 36.5; MS (ESI): 490.5 $[M+H]^+$.

Synthesis of compound 13. NaOH (2 M, 30 mL) was added to the solution of compound **3** (7.78 g, 0.02 mol) in DMF (35 mL). After stirring at 30 °C for 4 h, the mixture was acidified to pH 2-3 with concentrated hydrochloric acid, and partitioned between ethyl acetate and water. The organic phase was separated and washed with brine, dried over $MgSO_4$. Filtration and solvent evaporation gave buff oily compound. The compound was purified using column

chromatography (silica gel, $CHCl_3$/ MeOH/ Formic acid, 35/1/0.15) to give compound **13** (5.0 g, 99.13% purity) in 88% yield.

Mp: 164-165 °C; $[\alpha]_D^{23}$ -36 (*c* 1.1, MeOH); IR (KBr, cm^{-1}) 3377, 3254, 3023, 2913, 2852, 1710, 1635, 1600, 1537, 1419, 1241, 710; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 12.72 (s, 1H), 9.19 (s, 1H), 8.63 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.51 (t, *J* = 6.0 Hz, 1H), 7.44 (t, *J* = 7.2 Hz, 2H), 7.09 (d, *J* = 8.4 Hz, 2H), 6.64 (d, *J* = 8.4 Hz, 2H), 4.55-4.49 (m, 1H), 3.08-2.91 (m, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 173.4, 166.4, 155.9, 134.0, 131.4, 130.1, 128.3, 127.4, 115.0, 54.7, 35.5. HRMS (ESI): Calcd for $C_{16}H_{14}NO_4$ $[M-H]^-$ 284.0923, Found 284.0926.

Synthesis of compound 14. Benzoic acid and L-phenylalaninol were identified as starting materials, the synthetic procedures were similar with compound **5**.

Mp: 172-173 °C; $[\alpha]_D^{26}$ -89 (*c* 1.0, MeOH); IR (KBr, cm^{-1}) 3310, 1638, 1544, 1332, 1053, 1033, 697; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 8.19 (d, *J* = 8.4 Hz, 1H), 7.80-7.78 (m, 2H), 7.51-7.41(m, 3H), 7.26-7.23 (m, 4H), 7.17-7.14 (m, 1H), 4.86 (t, *J* = 5.6 Hz, 1H), 4.19-4.14 (m, 1H), 3.54-3.40 (m, 2H), 2.98-2.77 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 166.1, 139.5, 134.8, 131.0, 129.1, 128.2, 128.1, 127.3, 125.9, 62.9, 54.8, 53.3, 36.5; MS (ESI): 278.1 $[M+Na]^+$.

ASSOCIATED CONTENT

Supporting information

Additional characterization date of impurities **A**-**F**, compounds **3**, **5**, **6**, **13**, **14**, **Y101** and stereoisomers **Y101-(A-C)**. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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