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Identification, synthesis and characterization of novel Ensifentrine impurities

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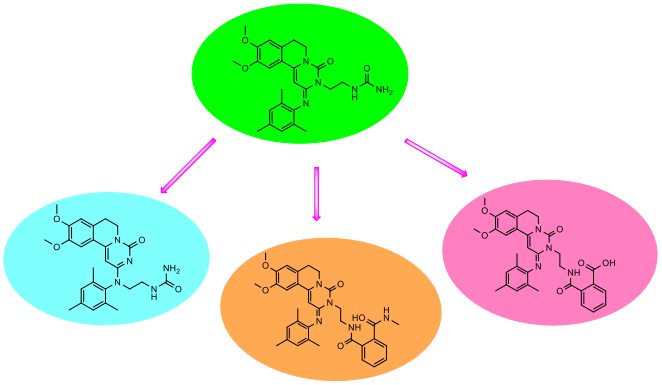
Abstract

Ensifentrine is a novel active pharmaceutical ingredient used in alleviation of respiratory ailments such as asthma and chronic obstructive pulmonary disease (COPD). In this work, three previously unreported impurities of Ensifentrine (ENF) namely, N-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4H-pyrimido[6,1-a]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}urea, N1-{2-[(2E)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2H-pyrimido[6,1-a]isoquinolin-3(4H)-yl]ethyl}-N2-methylbenzene-1,2-dicarboxamide, and 2-({2-[(2E)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2Hpyrimido[6,1-a]isoquinolin-3(4H)-yl]ethyl}carbamoyl)benzoic acid were detected while using high-performance liquid chromatography during the synthesis of ENF, in multiple batches between concentration range of 0.10 and 0.15%. These impurities were individually synthesized, isolated and characterized.



Keywords: Ensifentrine impurities, novel ensifentrine analogues, drug-intermediates.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a multifaceted, chronic lung disease which restricts the airflow gradually and causes difficulty in breathing.[1] While bronchodilators, particularly long-acting muscarinic antagonists (LAMAs) and long-acting β_2-agonists (LABAs) are currently used for standardized management of COPD, affected individuals with upper eosinophil counts of>300 cells/µl, requires inhaled corticosteroids (ICSs) in addition, which helps in reducing inflammation.[2] However, due to limited efficacy and side effects induced after long term use, many of these medications provided difficulty in the disease management. Dual PDE3 and PDE4 inhibition, one impacting smooth muscle function and other regulating inflammation in airway smooth muscle, were observed to have good clinical results.[3]

Ensifentrine (ENF), chemically known as *N*-{2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}ureaand also known as Ohtuvayre (see Figure 1), is a dual PDE3/PDE4 inhibitor[4] as described in WO 00/58308 A1. It has both anti-inflammatory, bronchodilatory and antihypertensive vasodilatoractivity, employed in clinical interventions of asthma and COPD.[5,6] It is typically administered by inhalation in view of its efficacy in the therapeutic approach of respiratory disorders. Symptoms like airflow blockage and associated breathing difficulties as seen in COPD can be reduced by relaxation of bronchial muscles, modulation of airway inflammation, and restoration of mucociliary clearance through dual inhibition of PDE3 and PDE4 by Ensifentrine as a result of its synergistic therapeutic effect.[7,8] Owing to the beneficial effect of Ensifentrine, it underwent assessment in multiple Phase 2a and Phase 2b clinical trials in nebulized form on COPD patients in the moderate to severe stage.[9,10]

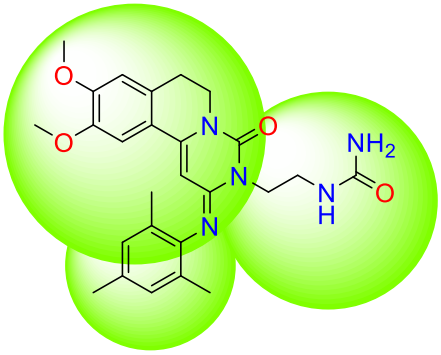


Figure 1. Chemical structure of Ensifentrine.

As reported previously, the suitable pharmaceutical composition for drug administration consists of Ensifentrine particles with coarse and fine lactose particles (Dv50 : 40 µm - 80 µm andDv50 : 5µm -10 µm, respectively), which is only up to 6.0 wt% of the total weight of the dry powder pharmaceutical composition[11] with maximum daily dosage of 3 mg BID of nebulized Ensifentrine.[12] It is presently undergoing Phase 3 clinical advancement as an emerging maintenance treatment for COPD, dosed two times in 24 hours using a jet-driven nebulizer.[13,14] Currently, Ensifentrine is the only novel therapeutic class of drug approved presently owing to its combined PDE3 and PDE4 inhibition, providing significant bronchodilation in monotherapy or synergistically with other FDA-recognized bronchodilators, as well as functioning broadly as NSAIDs.[15] It also enhances ciliary activity, which may alleviate sputum-related symptoms.[16] as well as its potential anti-pathogenic and antibiofilm activity against *Pseudomonas*

aeruginosa, highlighting an adjunctive therapy for COPD.[17] Also, novel salt forms and polymorphs of the model drug Ensifentrine (ENSE) with GRAS co-formers, revealed enhanced solubility and stability profiles offering critical insights for its formulation as a therapeutic agent.[18,19,6] The potential of inhaled Ensifentrine observed till date from its clinical and safety profile makes it a novel therapeutic approach for affected individuals with COPD.

Detecting and managing impurity profiles in drug substances and APIs is required for ensuring their pharmacological effect, as impurities can impact the drug's effectiveness, quality, toxicity and overall safety profile. So, it's important to isolate impurities that arise during the synthesis of active pharmaceutical ingredient (APIs) to meet the key criteria required for regulatory approval.[20] However, due to limited literature the impurity synthesis and characterization becomes difficult for the manufacturing units to access them. The generation and structural characterization of such impurities are difficult tasks that largely depend on synthetic pathways and reaction conditions used in the manufacturing process. Ensifentrine was synthesized in 2004 by Verona Pharma PLC following the synthetic scheme in S1.[20,21] Later, this methodology, with minimal changes in the reagents and reaction conditions, were also adapted for full Ensifentrine synthesis.

According to the available literature, several impurities were identified during this Ensifentrine synthesis (see Figure 2), but information about their individual synthesis and characterization was not reported. Three impurities, each present at levels ranging from 0.10-0.15% were detected in the final product obtained during R&D and pilot-scale synthesis using HPLC. These impurities were identified as, ENF N-alkyl substitution impurity (N-[2-({(2E)-9,10-dimethoxy-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2H-pyrimido[6,1-a]isoquinolin-4-yl}oxy)ethyl]urea), ENF N-methyl amide impurity (N^1-{2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}-N^2-methylbenzene-1,2-dicarboxamide) and ENF carboxylic acid impurity (2-({2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}carbamoyl)benzoic acid) after conducting LC-MS analysis. The study of these impurities can be helpful in the development of both manufacturing processes and formulations of Ensifentrine with minimal impurities, considering these unwanted by-products as calibration reference. Thereby, this study reports the synthesis and structural elucidation of three key API impurities of Ensifentrine that have been reported.

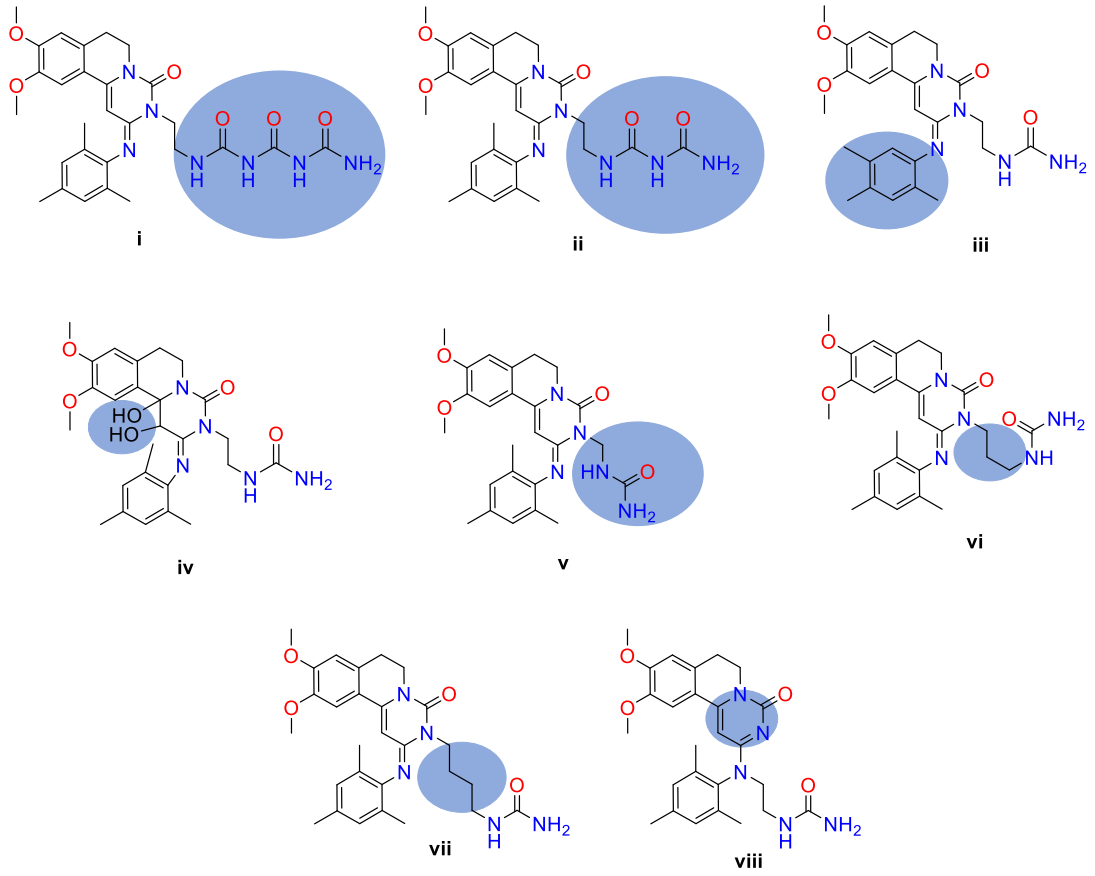


Figure 2. Chemical structures of Ensifentrine impurities as described in the literature.

Results and Discussion

During laboratory scale syntheses of Ensifentrine, batches were monitoring by HPLC during we observed two impurities forming at 1.8 RRT and 1.9 RRT in amounts from 0.10% to 0.15%. In order to identify the unidentified by-products formed during process development of Ensifentrine, LC–MS/MS spectrum and IR spectrum of the by-products were recorded. For the N-methyl amide impurity, the mass obtained in the +ve mode was 596.4 with IR 3288 (N-H stretching), whereas for ENF carboxylic acid impurity, the mass obtained in the +ve mode was 583.2 and IR 3330 (N-H stretching). The mass of ENF N-alkyl impurity in the +ve mode was 478.1 with IR 3469 (N-H stretching). Based on the mass spectral fragmentation and IR interpretation, we expected the unidentified by-products to be the ENF N-methyl amide, ENF Carboxylic acid and ENF N-alkyl impurities (see Figure 3).

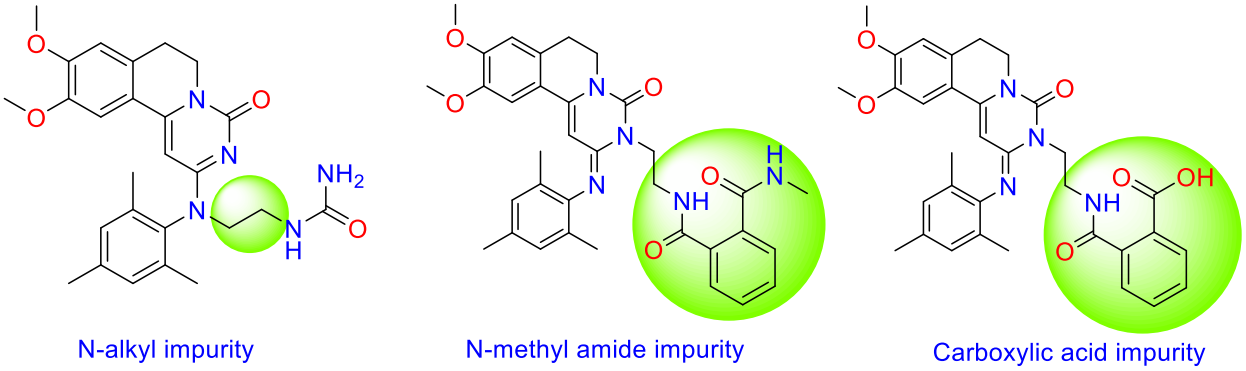
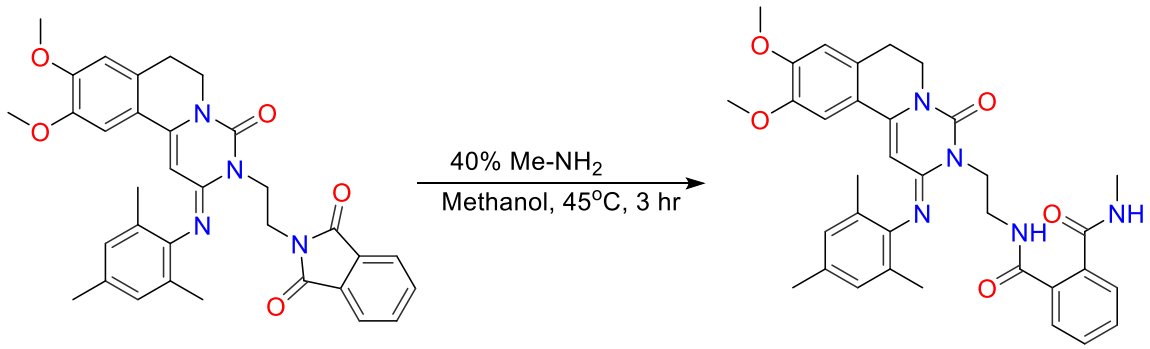


Figure 3. Identified impurities found during the process development of Ensifentrine.

In order to synthesize the three impurities, ENF N-methyl amide, ENF Carboxylic acid and ENF N-alkyl impurities, on larger scale, two single step processes and a multi-step process were carried out under standard conditions (Scheme 1-3). For the synthesis of the amide impurity, 40% methylamine was used in methanol to react with the phthalimide intermediate of ENF to produce ENFN-methyl amide under conditions as in Scheme 1.



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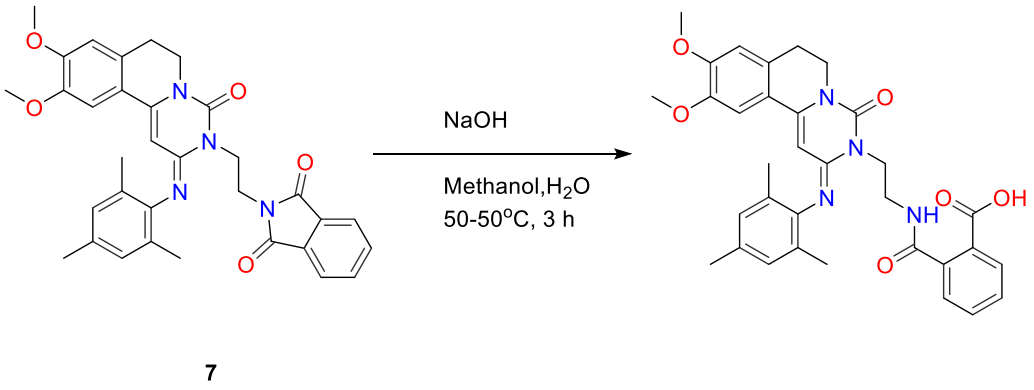
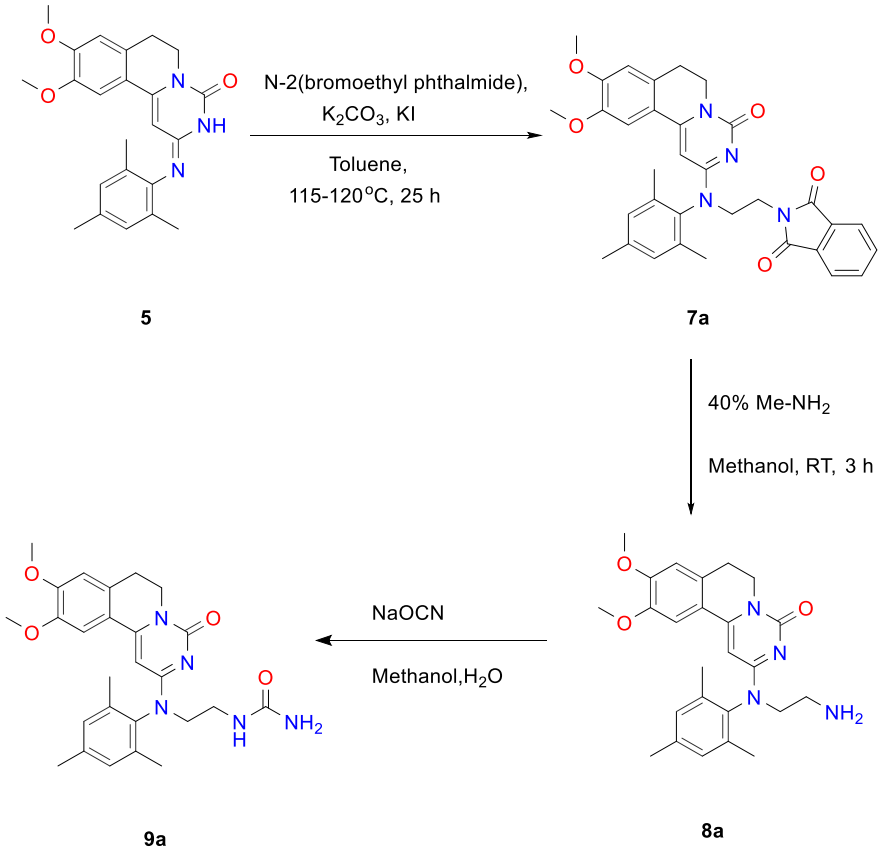
Scheme 1. Formation pathway of the ENF N-methyl amide impurity.

While synthesizing Ensifentrine, deprotection of phthalimide compound (7) was performed in 40% methyl amine in methanol. During this reaction, the formation of a carboxylic acid impurity was observed in the range 0.10-0.15% which suggested its formation being favoured under either acidic or basic conditions. To investigate this, separate standardization at acidic and basic environment was done. Different concentrations of NaOH were used as a base in presence of methanol and water to produce ENF carboxylic acid under standard conditions as in Scheme 2.



Scheme 2. Formation pathway of ENF Carboxylic acid Impurity.

We synthesized ENF-N-Alkyl substituted impurity following a multi-step process where firstly we did N-phthalimidation of ENF Trequisin in Toluene to produce N-phthalimide substituted ENF Trequisin. In the next step, amination reaction was carried out by 40% methyl amine in Methanol with phthalimide as the leaving group to produce N-ethylamine substituted analog of ENF Trequisin. In the last step we used sodium cyanate in methanol and water to react with N-ethylamine substituted analog of ENF Trequisin to produce N-ethyl urea substituted impurity of ENF Trequisin. (Scheme 3). The N-alkylated impurity structure was also confirmed from the crystal data as in Figure 4.[22]



Scheme 3. Formation pathway of N-alkyl impurity.

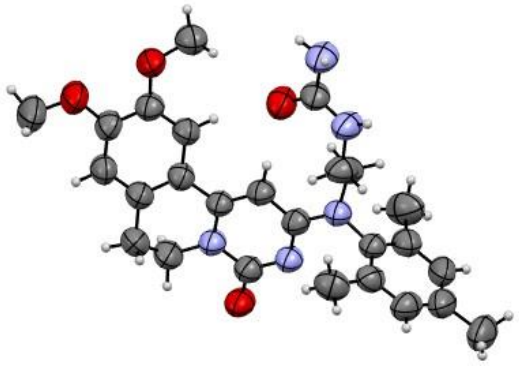


Figure 4. Single Crystal Structure: ENF N-alkyl Impurity (CCDC no : 2422551).

Several optimization conditions were explored to effectively minimize the formation of these impurities during manufacturing. For controlling N-alkyl impurity, we incorporate chlorobenzene solvent with toluene (1:1) and so maximum N-substituted isomer formed. (see S1, step 5). To remove N-methyl amide impurity, we used ACN + water purification (see S1, step 6), and in order to suppress the generation of Carboxylic acid by-product, we raised the temperature of the reaction up to 45 °C which controlled impurity formation which was then further removed in ACN + water purification. All impurities were characterized by proton (comparison see Table 1), mass spectrometry and IR spectroscopy – see the Supplementary Information file for more detail.

Conclusions

Three compounds, named the N-alkyl isomer, N-methyl amide and N-carboxylic acid, were confirmed as by-products isolated from multiple synthetic batches of Ensifentrine. The structure of these compounds were confirmed through various analytical techniques. Secondly, the compounds were effectively synthesized on laboratory-scale using readily accessible drug-intermediates. The compounds can serve as valuable reference standards for pharmaceutical organizations and research institutions, aiding in the identification and control of impurity formation during manufacturing process. This study holds significant relevance for researchers engaged in the process and formulation development of Ensifentrine API.

Experimental Section

General. The samples used in this study were synthesized in Chemsar Laboratory. ENF N-alkyl Ensifentrine impurity, ENF N-methyl amide impurity and ENF Carboxylic acid impurity were synthesized and then purified using preparative HPLC if required. All the HPLC- grade solvents, NMR solvents, and reagents were procured from Merck Life Sciences, India.A Waters (LC2695) HPLC system was used for the present study using a PDA-2996 detector set at 260 nm. Empower 2 software was used to process the HPLC data. The Epic C-18 (PerkinElmer, 250 mm × 4.6 mm, 5 μm) analytical column was used to perform the analysis. Mobile phase A was 10 mM ammonium acetate, and mobile phase B was methanol/acetonitrile (50:50, v/v). The linear gradient program was set as follows: Tmin/B(mL/min); T 0/10; T15/ 90; T25/90; T26/10; T30/10. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. The homogeneous mixture of HPLC-grade water

and HPLC- grade methanol in the ratio 1:1 was used as a diluent for sample preparation.LC−MS analysis of the degraded sample of Ensifentrine was done on a Waters 2695 (Water Corporation) ACQUITY HPLC-MS system. The EPIC C18 column (4.6 × 150 mm, 3 μm) was used as an analytical column for chromatographic separation. The wavelength of the UV detector was set at 260 nm. Mobile phase A consisted of 0.1% formic acid. Mobile phase B consisted of HPLC-grade acetonitrile. The flow rate was set at 0.8 mL per min, and the column oven temperature was set at 25 °C. The cone voltage was 30 V, and the capillary voltage was 3.5 kV. The source temperature was maintained at 120 °C. Nitrogen gas was used as both the desolation and cone gases with flow rates of 350 L/h and 50 L/h, respectively. The linear gradient program was set as follows: Tmin/B (%) T 0/10; T15/90; T25/90; T26/10; T30/10.Purification of ENF carboxylic acid impurity was done from the enriched samples obtained from the reaction of deprotection of ENF phthalimide compound (7) using the described procedure mentioned in Scheme 1. The required impurity peak was isolated using the Waters 2545 preparative HPLC system equipped with the Phenomenex C18 column (250 × 20 mm, 10 μm), and the PDA2996 detector was set at 260 nm. Mass Lynx software was used to process the data. Ammonium and the run time was 40 min. The linear gradient program was set as follows: Tmin/B(mL/min): T0/20; T5/30; T15/40; T20/50; T25/80; T35/90; T40/100. The ENF carboxylic acid impurity obtained from the preparative HPLC fraction was purified heat-cool, heat-cool method in acetonitrile to get pure desired impurity as a pale-yellow solid. The isolated sample was further used for its complete characterization. NMR spectra were recorded on a Bruker Avance 400 spectrometer (^{1}H at 400 MHz and ^{13}C at 100 MHz). Chemical shifts for ^{1}H NMR spectra are reported in parts per million (ppm) relative to tetramethylsilane, with the solvent resonance as the internal standard ($CDCl_3$: δ 7.26 ppm). Similarly, ^{13}C NMR spectra are reported in ppm relative to tetramethylsilane, using the solvent as the internal standard ($CDCl_3$: δ 77.0 ppm). For more characterization, including 2D spectroscopic results, see the Supplementary Information file.

ENF N-methyl amide impurity, (*N*^{1}-{2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}-*N*^{2}-methylbenzene-1,2-dicarboxamide): 10 g Phthalimide compound (7) was taken in 70 ml of methanol. To that 40% aqueous methyl amine solution was added at room temperature. Reaction mass was stirred for 4 hours at 45 °C. Reaction mass was then quenched by 40 ml Conc. HCl. Formed precipitate was filtered and washed with 10 ml methanol. Filtrate was distilled and residue was purified by flash column chromatography to obtain 0.2 g impurity with good purity.
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (s, 1H), 8.07 (s, 1H), 7.46 (dd, *J* = 8.2Hz, 2.1 Hz, 4H), 6.96 (s, 1H), 6.85 (s, 2H), 6.67 (d, 1H), 5.34 (s, 1H), 4.34 (t, *J* = 6.4Hz, 2H), 3.93 (t, *J* = 6.8Hz, 2H), 3.80 (s, 3H), 3.61 (broad hump, 5H), 2.90 (t, *J* = 6.8Hz, 2H), 2.70 (d, 3H), 2.22 (s, 3H), 1.98 (s, 6H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 168.9, 168.6, 152.1, 151.7, 148.6, 144.5, 142.7, 136.7, 136.7, 131.0, 130.7, 129.7, 129.7, 129.6, 128.8, 128.8, 128.2, 128.2, 128.0, 119.4, 111.8, 109.0, 88.3, 56.5, 41.3, 40.0, 37.1, 27.5, 26.5, 20.9, 18.5. IR**:** 3288 (N-H Stretching), 2937, 2911,1636, 1511, 1474, 1436, 1289, 1268, 1033, 986. *m/z* $(M + H)^+$ calc for $C_{34}H_{38}N_5O_5$, 595.7002; found 596.2761.

ENF Carboxylic acid Impurity, (2-({2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}carbamoyl)benzoic acid): Sodium hydroxide in methanol and water (9:1) was added to phthalimide compound (7) in methanol and treated at higher temperatures to obtain Carboxylic acid impurity in good yield. The maximum yield of impurity was observed by using NaOH (2 eq.) in methanol and water (5 vol.) as a solvent at 50-55 °C for 4 h. The crude carboxylic acid impurity was further purified by preparative HPLC in Section 2. Reaction Scheme: 2-({2-[(2*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-3(4*H*)-yl]ethyl}carbamoyl)benzoic acid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.67 (d, *J* = 8.2Hz, 1H), 7.48 (d, *J* = 8.2Hz, 1H),

7.32 (t, *J* = 8.4Hz, 1H), 7.24 (t, *J* = 8.4Hz, 1H), 6.96 (s, 1H), 6.85 (s, 2H), 6.67 (d, 1H), 5.33 (s, 1H), 4.30 (t, *J* = 6.4Hz, 2H), 3.92 (t, *J* = 6.8Hz, 2H), 3.80 (s, 3H), 3.61 (s, 5H), 2.91 (t, *J* = 6.8Hz ,2H), 2.21 (s, 3H), 1.97 (s, 6H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 172.9, 168.7, 152.1, 151.4, 148.5, 144.6, 142.7, 142.6, 132.9, 131.1, 130.6, 129.6, 129.1, 129.1, 128.8, 128.8, 128.1, 128.1, 126.9, 119.4, 111.8, 109.0, 88.1, 56.5, 56.1, 41.2, 40.0, 36.7, 27.5, 20.9, 18.4, 18.4. IR: 3330 (N-H Stretching), 2935, 2910, 1636, 1565, 1510, 1159, 1111, 1031, 986. *m/z* (M + H)$^+$ calc for $C_{33}H_{35}N_4O_6$, 582.6570; found 583.2457.

ENF N-Alkyl substitution impurity, (*N*-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}urea[1]

Stage-1: Synthesis of 2-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}-1*H*-isoindole-1,3(2*H*)-dione (Synthesis of N-alkyl phthalimide) (7a)[23]

(2*E*)-9,10-dimethoxy-2-[(2,4,5-trimethylphenyl)imino]-2,3,6,7-tetrahydro-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one (10 g, 25.54 mmol) (**5**), was taken into 50 ml chlorobenzene. To that N-(2-bromoethyl)phthalimide (20 g, 3.08 equiv), Sodium iodide (8.0 g, 2.08 equiv) and Potassium carbonate powder 40 mesh (20 g, 5.7 equiv) were added and reaction mass was heated for 5 hours at 115-120 °C. Then the reaction mass 50 ml toluene was added and the content was heated at 105-110 °C for 20 hours. Reaction mass was then cooled to 55-60 °C and to that 2 g hyflo bed was added. Reaction mass was stirred for 1 hr at 25-30 °C and then filtered. Given material was adsorbed on hyflo bed. Hyflo bed material was taken into 500 ml RBF. 200 ml MDC: methanol (8:2) was added into RBF. The RM was stirred at 35-40 °C for 1 hr. RM was then cooled to 25-30 °C and stirred for 1 hr. RM was then filtered and washed with 20 ml MDC. Filtrate was distilled under vacuum to get off white material. Isolated material was further purified in 50 ml toluene by heat cool- heat cool method at 55-60 °C. Obtained material was taken in 50 ml methanol and stirred for 1 hour at 40-45 °C and then cool, filtered to afford 4 g pure ENF N-Alkyl phthalimide compound 7a. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.84 (dd, *J* = 8.2Hz, 2.1 Hz, 2H), 7.72 (dd, *J* = 8.2Hz, 2.1 Hz , 2H), 6.99 (s, 2H), 6.72 (s, 1H), 6.67 (s, 1H), 5.39 (s, 1H), 4.24 (t, *J* = 6.4Hz, 2H), 4.18 (t, *J* = 6.8Hz , 2H), 3.99 (t, *J* = 6.4Hz, 2H), 3.93 (s, 3H), 3.75 (s, 3H), 2.91 (t, *J* = 6.8Hz , 2H), 2.32 (s, 3H), 2.22 (s, 6H).^{13}C NMR (500 MHz, DMSO-d_6) δ 167.9, 163.4, 157.1, 152.0, 150.0, 148.1, 138.1, 136.7, 136.5, 133.8, 132.0, 130.5, 129.8, 123.1, 119.9, 110.4, 108.8, 86.3, 56.3, 56.0, 46.3, 40.0, 36.1, 27.6, 20.9, 18.2. IR: 3080, 3002, 2939, 2918, 1769, 1703, 1654, 1510, 1462, 1154, 1130, 1066, 1036 (C-N Stretching).*m/z* (M + H)$^+$ calc for $C_{33}H_{33}N_4O_5$, 564.6424 u; found 565.5532 u.

Stage-2: Synthesis of 2-[(2-aminoethyl)(2,4,6-trimethylphenyl)amino]-9,10-dimethoxy-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one. (Synthesis of N-alkyl amine) (8a)[23]

2-[2-({(2*E*)-9,10-dimethoxy-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-yl}oxy)ethyl]-1*H*-isoindole-1,3(2*H*)-dione (4 g, 7.092 mmol) (**7a**) was diluted in 7 vol methanol. To that 14 ml 40% methyl amine was added and reaction mass was stirred at 40-45 °C. After 4 hours reaction mass was cooled to 0-5°C and then pH of reaction mass was adjusted to 1-2 by using Conc HCl. After 1 hour stirring at cool temperature reaction mass was filtered and washed with 10 ml methanol. Obtained precipitate was purified in ACN: Water (3 vol :0.3 vol) at 50-55°C and then filtered at RT affording white compound with good yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.07 (s, 2H), 6.98 (s, 1H), 6.58 (s, 1H), 5.23 (s, 1H), 3.93 (m, 2H), 3.81 (s, 3H), 3.73 (t, *J* = 6.4Hz, 2H), 3.59 (s, 3H), 2.88 (m, 2H), 2.77 (m, *J* = 6.4Hz, 2H), 2.30 (s, 3H), 2.11 (s, 6H).^{13}C NMR (500 MHz, DMSO-d_6) δ 162.6, 155.7, 151.9, 149.5, 147.7, 137.3, 137.2, 135.8, 130.7, 129.5, 119.0, 111.3, 108.5, 84.8, 55.8, 55.7, 51.7, 40.0, 39.0, 26.7, 20.5, 20.4, 17.6. IR: 3366, 3288 (N-H Stretching), 2997, 2919, 1734, 1636, 1503, 1459, 1178, 1145, 1079, 1049, 1031 (C-N Stretching).*m/z* (M + H)$^+$ calc for $C_{25}H_{31}N_4O_3$, 434.1235 u; found 435.1235 u.

Stage-3: Synthesis of *N*-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}urea (Synthesis of ENF N-alkyl Ensifentrine) (9a)[23]

2-({(2*E*)-9,10-dimethoxy-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-yl}oxy)ethan-1-amine (1 g, 2.30 mmol) (**8a**) was taken in 30 v methanol. Reaction mass was heated to 65-70 °C. Sodium cyanate solution (0.25 g, 1.8 equiv in 5 ml water) was added slowly into the reaction mass. After 1 hour reaction mass was cooled to 25-30 °C and to that 10 ml water was added. Reaction mass was then stirred for 1 hour and filtered to get white solid material. Purification was done in MDC: Methanol: EA (10: 1.5: 15) at room temperature to afford white solid material **9a**.^{1}H NMR (500 MHz, DMSO-d_6) δ 7.78 (s, 1H), 7.27 (s, 1H), 7.06 (s, 1H), 6.98 (s, 1H), 6.93 (s, 1H), 6.30-6.21 (broad hump, 1H), 5.70 (s, 1H), 5.58 (broad hump, 1H), 3.97 (t, *J* = 6.4Hz, 2H), 3.94 (t, *J* = 6.8Hz, 2H), 3.84 (s, 3H), 3.70 (m, 1H), 3.58 (s, 1H), 3.53 (m, 1H), 3.25 (t, *J* = 6.8Hz, 2H), 2.89 (t, *J* = 6.4Hz, 2H), 2.29 (s, 3H), 2.10 (s, 6H).^{13}C NMR (500 MHz, DMSO-d_6) δ 163.1, 162.6, 159.4, 155.5, 152.0, 151.4, 150.5, 148.1, 147.7, 139.7, 137.4, 137.2, 135.8, 135.7, 134.9, 130.7, 129.8, 129.6, 128.9, 119.6, 118.9, 111.3, 110.7, 109.3, 108.6, 85.2, 84.9, 56.3, 55.8, 55.7, 55.6, 50.9, 49.2, 37.8, 26.8, 26.7, 20.5, 20.4, 17.9, 17.6. IR: 3469 (N-H Stretching), 2996, 2953, 1637, 1596, 1563, 1269, 1218, 1157, 1115 (C-N Stretching).*m/z* $(M + H)^+$ calc for $C_{26}H_{32}N_5O_4$, 477.5651u ; found 478.2369 u.

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Supplementary Material

Copies of the ^{1}H and ^{13}C NMR spectra of all compounds are given in the supplementary material file associated with this manuscript.

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