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Identification, Synthesis, and Characterization of Potential Oxidative Impurities of Venetoclax: Application of Meisenheimer Rearrangement

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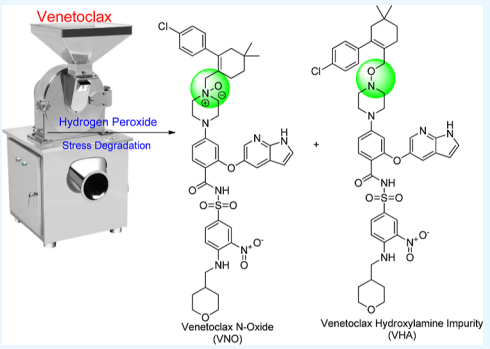
ABSTRACT: Venetoclax is a potent BCL-2 inhibitor that is used for the treatment of several blood cancers. During the oxidative stress degradation of venetoclax, we observed the formation of two potential impurities at levels of about 8−10%, which have similar molecular weights. The two impurities were isolated and identified as 4-(3-((1*H*-pyrrolo[2,3-*b*]pyridin-5-yl)oxy)-4-(((3-nitro-4-(((tetrahydro-2*H*-pyran-4-yl)methyl)amino)phenyl)sulfonyl)carbamoyl)phenyl)-1-((4′-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)methyl)piperazine 1-oxide (venetoclax *N*-oxide, **VNO**) and 2-((1*H*-pyrrolo[2,3-*b*]pyridin-5-yl)oxy)-4-(4-((4′-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)methoxy)-piperazin-1-yl)-*N*-((3-nitro-4-(((tetrahydro-2*H*-pyran-4-yl)methyl)amino)-phenyl)sulfonyl)benzamide (venetoclax hydroxylamine impurity, **VHA**). To confirm these two compounds, we have synthesized each impurity individually and analyzed it by high-performance liquid chromatography, mass spectrometry, ^{1}H NMR, ^{13}C NMR, and 2D NMR. VNO was synthesized by the oxidation of venetoclax using *m*-CPBA in dichloromethane to get the required *N*-oxide impurity. After the confirmation of the VNO impurity, the VNO impurity was heated with water at reflux in a sealed tube for 36 h to get the VHA impurity of about 6−8% after 36 h. After thorough analysis, it was confirmed that venetoclax *N*-oxide undergoes [1,2] Meisenheimer rearrangement to form the venetoclax hydroxylamine impurity. These two impurities may be relevant reference standards in manufacturing venetoclax Active Pharmaceutical Ingredient (API) (or) tablets.



1. INTRODUCTION

Venetoclax is one of the potent drugs used for the treatment of various blood cancers. Venetoclax **1** (Venclexta) is chemically known as (4-[4-[[2-(4-chlorophenyl)-4,4-dimethylcyclohexen-1-yl]methyl]piperazin-1-yl]-*N*-[3-nitro-4-(oxan-4-ylmethylamino)phenyl]sulfonyl-2-(1*H*-pyrrolo[2,3-*b*]pyridin-5-yloxy)benzamide) and is a Bcl-2 (B cell lymphoma-2) (Figure 1) selective inhibitor.[1,2]

Chemically, venetoclax contains a biaryl acylsulfonamide core of the anticancer molecule. Venetoclax is used in combination with azacitidine and decitabine for the treatment of acute myeloid leukemia for adults who are aged above 75 years. Venetoclax was approved by the US FDA in 2015 for the treatment of CLL and SLL. In 2016, the European Union approved it for the treatment of CLL and SLL.[3]

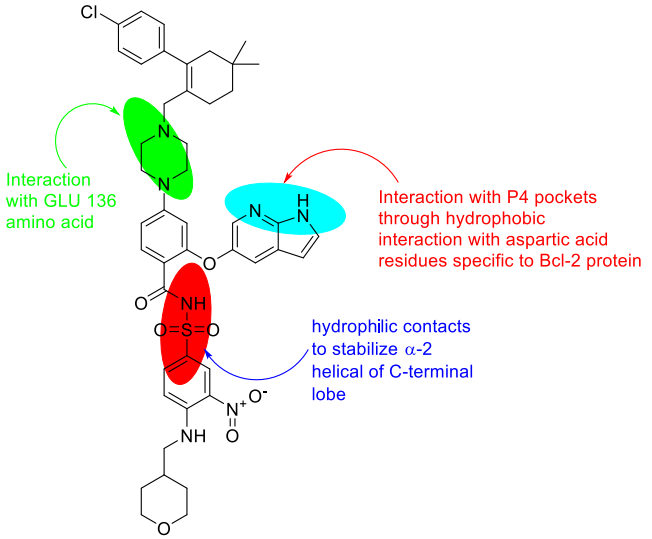


Figure 1. Chemical structure of venetoclax **1**.

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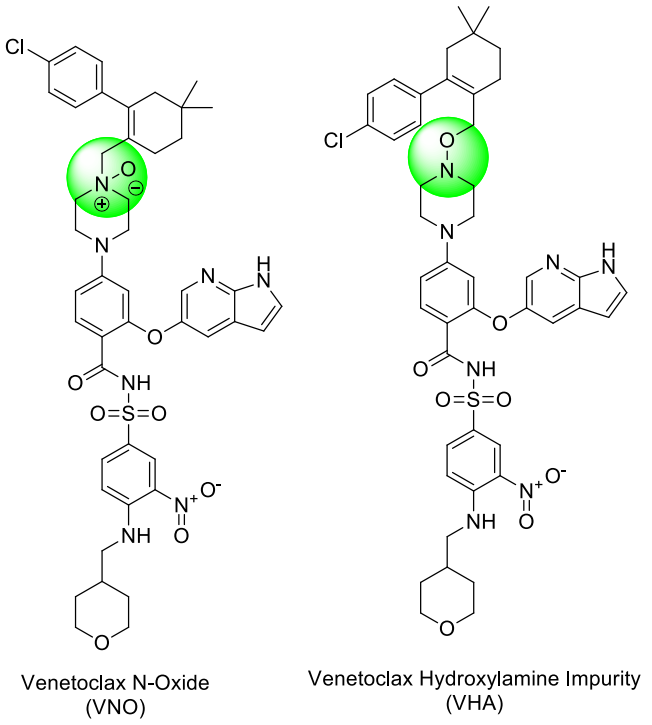


Figure 2. Chemical structures of Venetoclax impurities.

The synthesis of anticancer compounds with a high safety profile has become an important task for researchers. To prepare high safety drugs, it is important to control the impurities during the synthesis as well as during the supply of the drug. It is very important to find the unknown impurities that are formed during the supply and storage of the drug. For this, forced degradation studies will be used to identify and minimize the formation of unknown impurities. A forced degradation study is carried out at severe conditions when compared with accelerated conditions to evaluate the molecule stability.[4,5]

As per the ICH (International Council for Harmonization) guidelines, to file a dossier registration, it is mandatory to perform stress studies of the drug.[6,7] These degradation studies will help find the suitable conditions for the storage.[8,9] As per the guidelines established by the ICH tripartite, the specified minimum level for reporting impurities in new drug substances is 0.05%, while the minimum level for their identification is 0.10%, considering a maximum daily dose of ≤2 g/day.[10]

On the other hand, Meisenheimer rearrangement is thermal rearrangement of *N*-oxides of tertiary amines to the corresponding hydroxylamines.[11] Meisenheimer rearrangement proceeds via a [1,2]- or [2,3]-rearrangement to obtain the rearranged product.

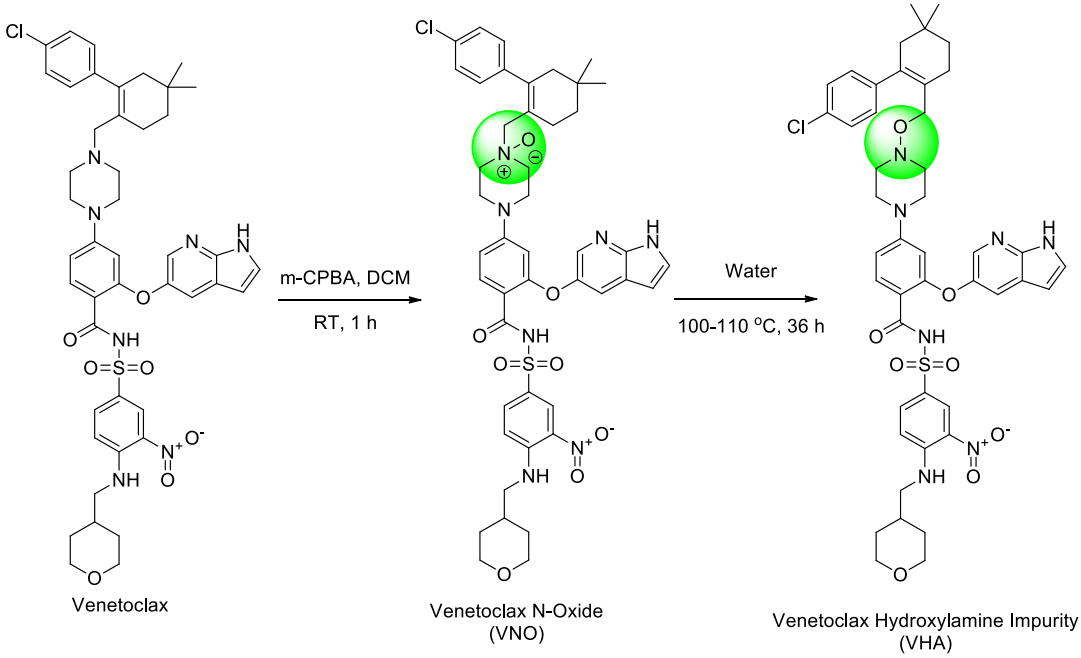
In this paper, we subjected venetoclax for oxidative stress using a 10% hydrogen peroxide solution. We obtained two major impurities (Figure 2) during the stress conditions, and these two impurities were thoroughly characterized to identify their structure owing to our interest toward the identification of unknown impurities and API impurities.[12−15]

In the recent literature, venetoclax was subjected to the stressed degradation studies to get several impurities, which were analyzed by liquid chromatography tandem mass spectroscopy (LC−MS/MS) analysis.[16] To understand the oxidative stress thoroughly, we report the identification, synthesis, and structural illustration of two major impurities of venetoclax obtained during the oxidative degradation of venetoclax in this article.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents. All of the compounds used in this study were synthesized in SVAK Life Sciences. Venetoclax *N*-oxide and venetoclax hydroxyl amine impurities were synthesized and then purified using preparative high-performance Liquid chromatography(Prep-HPLC) if needed.

Scheme 1. Synthesis of Venetoclax *N*-Oxide and Venetoclax Hydroxylamine Impurities



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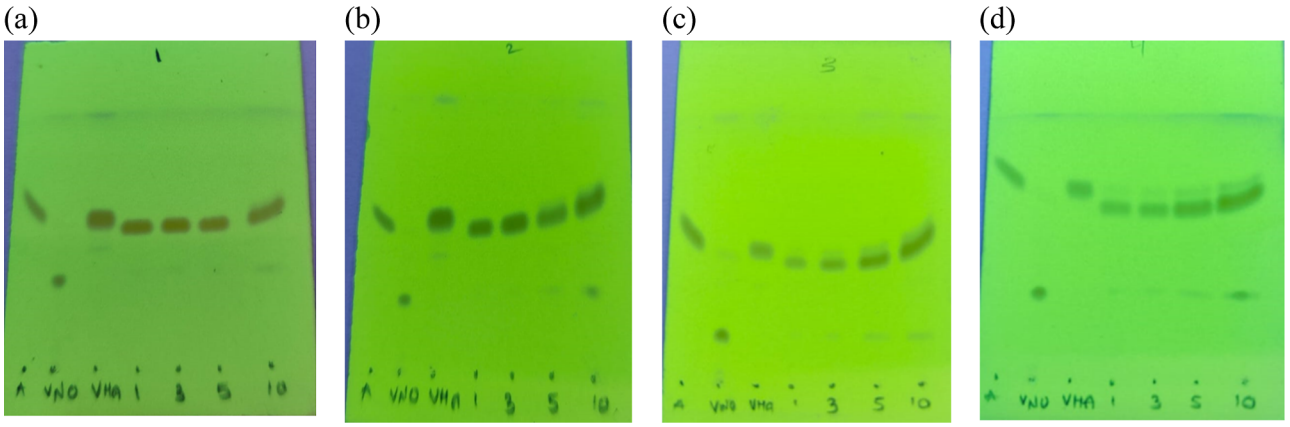

Figure 3. TLC chromatogram of the venetoclax stress degradation sample by using 1, 3, 5, and 10% H_2O_2 solutions. **A**—venetoclax API, **VNO**—venetoclax *N*-oxide, and **VNH**—venetoclax hydroxylamine impurity; (a) after 1 h, (b) after 2 h, (c) after 3 h, and (d) after 4 h.

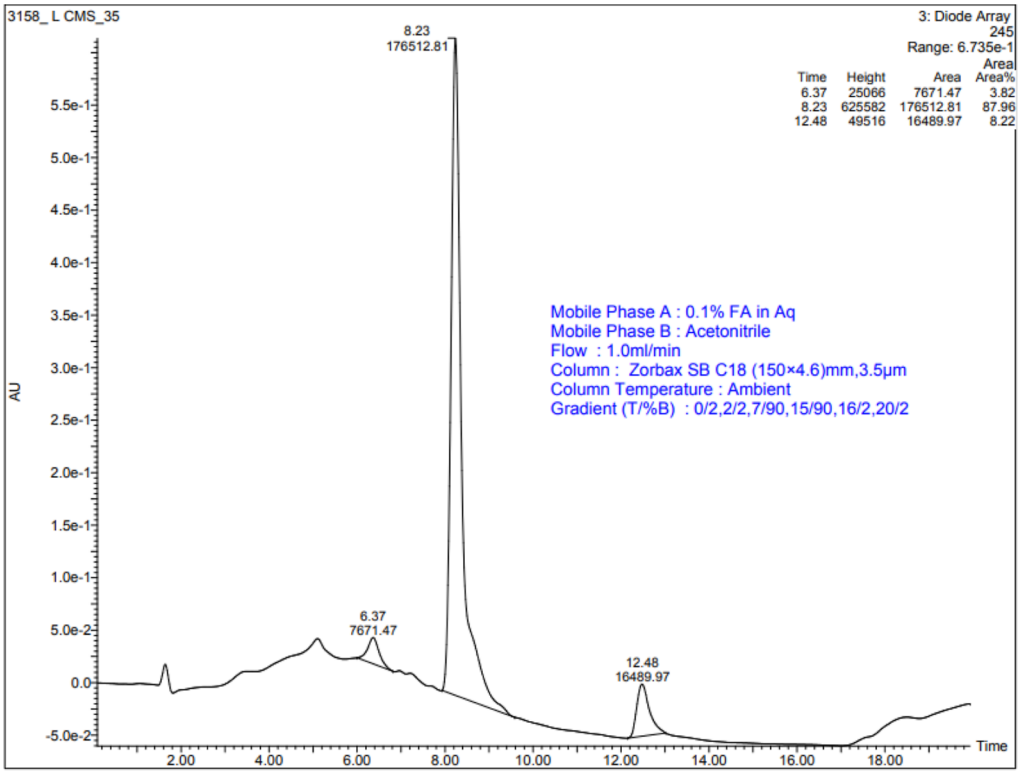

Figure 4. LC−MS chromatogram of the venetoclax stress degradation sample.

All the HPLC grade solvents, NMR solvents, and reagents were procured from Merck Life Sciences, India.

2.2. High-Performance Liquid Chromatography (Analytical). A Waters (LC2695) HPLC system was used for the analysis of venetoclax impurities using a PDA-2996 detector set at 245 nm. To process the HPLC data, Empower 2 software was used. A Phenomenex Luna C18 column (50 × 250 mm, 10 μm) was used to perform the HPLC analysis. Mobile phase A was 0.1% aqueous trifluoroacetic acid, and mobile phase B was acetonitrile. The linear gradient program was set as follows: Tmin/B(mL/min): T0/10; T15/90; T25/90; T26/10; and T30/10. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. Acetonitrile was used as a diluent for the sample preparation.

2.3. Liquid Chromatography−Mass Spectroscopy. An oxidative stress sample of venetoclax was analyzed using a Waters 2695 (Water Corporation) ACQUITY HPLC-MS system. The Zorbax SB C-18 column (4.6 × 150 mm, 3.5 μm) was used for chromatographic separation (Figures S16−S19). The wavelength of the UV detector was set at 245 nm. Mobile phase A is 0.1% formic acid in water, and mobile phase B is HPLC grade acetonitrile. The flow rate was set at 1.0 mL/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. The linear gradient program was set as follows: Tmin/B(%): T0/02; T02/02; T07/90; T15/90; T16/02; and T20/02.

2.4. High-Performance Liquid Chromatography (Preparative). Purification of the venetoclax impurity was done from the enriched samples obtained from the oxidative stress samples of venetoclax using the procedure mentioned in Section 2.5. A Waters 2545 preparative HPLC system with a

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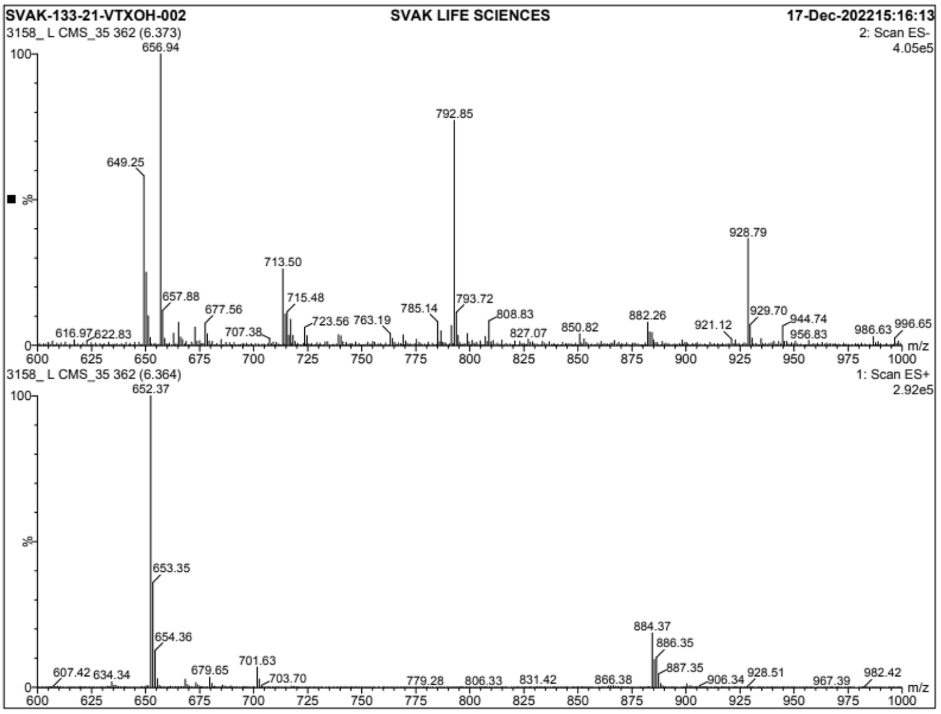

Figure 5. Mass spectrum of venetoclax *N*-oxide.

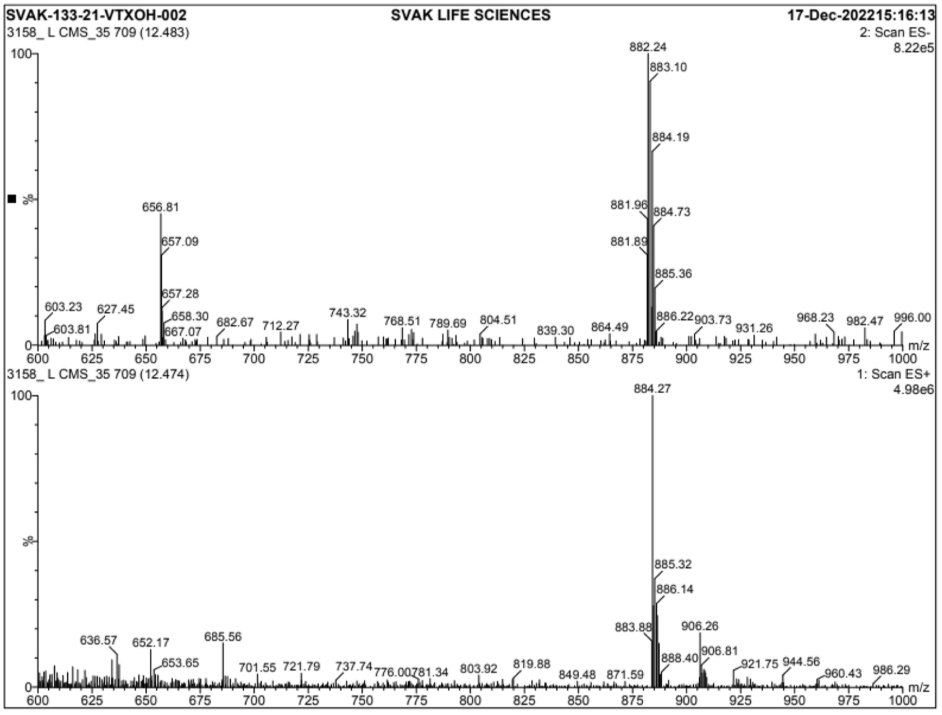

Figure 6. Mass spectrum of the venetoclax hydroxylamine impurity.

Phenomenex Luna C18 column (50 × 250 mm, 10 μm) and a PDA2996 detector was used to isolate the required impurity peak (Figure S15). The wavelength was set to 245 nm. To process the data, MassLynx software was used. 0.1% aqueous trifluoroacetic acid was used in mobile phase A, and a 1:1 mixture of HPLC grade acetonitrile and methanol was used in mobile phase B. The flow rate was maintained at 30 mL/min, and the run time was 100 min. The linear gradient program was set as follows: Tmin/B(mL/min): T0/40; T3/50; T5/60; T09/70; T13/70; T18/75; T30/75; T43/80; T50/90; and T60/100. The VNO and VHA impurities obtained from the preparative HPLC fraction were extracted with 100 mL of MeOH−DCM (1:9), and the organic layer was concentrated to get VNO and VHA impurities as pale-yellow solids. The isolated samples were further used for its complete characterization.

2.5. Stress Studies. We performed different oxidative stress conditions as per ICH Q1A (R2) and ICH Q1B.[17,18]

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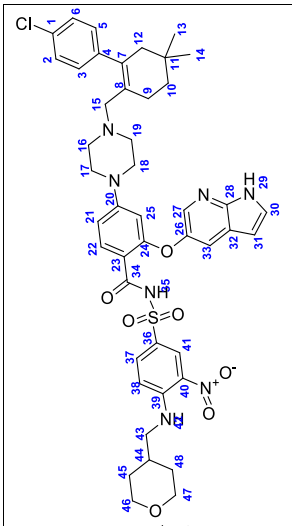
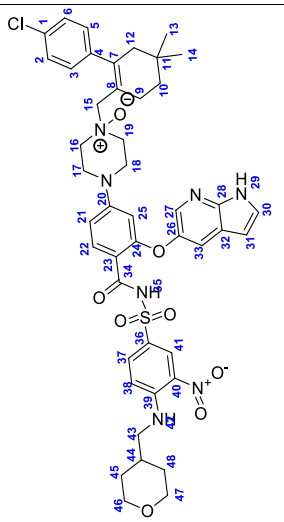
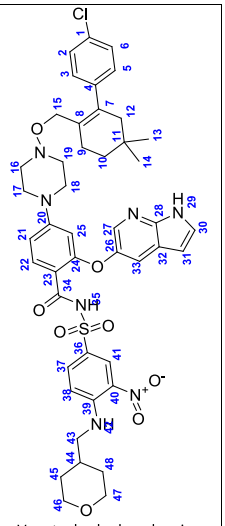
Table 1. ^{1}H NMR and ^{13}C NMR Assignment of Venetoclax, VNO, and VHA Impurities



venetoclax **1**



Venetoclax N-Oxide **2**



Venetoclax hydroxyl amine impurity **3**

Carbon number	^{1}H NMR ppm (Multiplicity)	^{13}C NMR ppm	^{1}H NMR ppm (Multiplicity)	^{13}C NMR ppm	^{1}H NMR ppm (Multiplicity)	^{13}C NMR ppm
1	-	130.8	-	130.1	-	131.1
2	7.33-7.36 (d, *J*=8.4 Hz)	128.1	7.37-7.39 (d, *J*=8.4 Hz)	127.7	7.34-7.36 (d, *J*=8.4 Hz)	127.9
3	7.03-7.12(m)	130.0	7.11-7.13 (m)	129.8	7.11-7.19 (m)	129.8
4		141.9		144.6		141.2
5	7.03-7.12(m)	130.0	7.11-7.13 (m)	129.8	7.11-7.19 (m)	129.8
6	7.33-7.36 (d, *J*=8.4 Hz)	128.1	7.37-7.39 (d, *J*=8.4 Hz)	127.7	7.34-7.36 (d, *J*=8.4 Hz)	127.9
7	-	134.6	-	134.9	-	135.2
8	-	128.5	-	128.7	-	128.6
9	2.14 (brs)	25.1	2.40 (m)	20.7	2.18 (brs)	25.3
10	1.36-1.40(t, *J*=6.4 Hz)	34.8	1.37-1.40 (t, *J*=6.4 Hz)	34.8	1.30-1.39 (t, *J*=6.4 Hz)	34.6
11	-	28.8	-	28.6	-	28.9
12	1.95 (s)	46.3	2.03 (s)	45.7	1.99 (s)	45.3
13	0.92 (s)	27.9	0.94 (s)	27.9	0.92 (s)	27.7
14	0.92 (s)	27.9	0.94 (s)	27.8	0.92 (s)	27.7
15	2.75 (m)	59.6	4.03 (brs)	70.0	3.84-3.89 (m)	71.2
16,19	2.19 (brs)	52.0	3.45-3.49 (m)	61.2	3.07 (brs)	54.2
17,18	3.07 (brs)	46.5	3.22-3.31 (m)	47.8	2.79 (brs)	45.5
17′,18′	3.07 (brs)	46.5	3.02-3.05 (m)	47.4	3.57 (brs)	45.5
16′,19′	2.19 (brs)	52.0	3.22-3.31 (m)	59.7	2.46 (brs)	54.2
20	-	154.5	-	151.3	-	154.3
21	6.67-6.69 (d, *J*=6.2 Hz)	108.7	6.62-6.65 (d, *J*=6.8 Hz)	109.4	6.72-6.75 (d, *J*=7.2 Hz)	108.9
22	7.48-7.53 (m)	132.1	7.29-7.30 (brs)	131.7	7.51-7.55 (m)	132.1
23	-	112.6	-	113.8	-	112.4
24	-	157.8	-	156.3	-	157.7
25	6.19 (brs)	102.4	6.30-6.32 (m)	105.4	6.26 (brs)	102.6
26		146.5		146.1		146.5
27	8.04 (d, *J*=2.4 Hz)	135.3	7.92-7.93 (d, *J*=2.4 Hz)	140.6	8.06 (d, *J*=2.8 Hz)	135.2
28	-	145.4	-	144.9	-	145.4
29	11.70 (s)	-	11.55 (s)	-	11.70(s)	-
30	7.48-7.53 (m)	127.7	7.42-7.44 (m)	127.2	7.51-7.55 (m)	127.7
31	6.39 (brs)	100.0	6.30-6.32 (m)	99.7	6.39-6.40 (brs)	99.9
32	-	119.8	-	121.8	-	119.7
33	7.48-7.53 (m)	117.9	7.55-7.57 (d, *J*=8.4 Hz)	119.6	7.51-7.55 (m)	117.7
34	-	163.8	-	169.0	-	163.4
35	11.32 (brs)	-	11.55 (s)	-	11.54 (s)	-
36		124.9		125.9		124.4
37	7.79-7.81 (d, *J*=9.2 Hz)	133.9	7.63-7.65 (m)	134.4	7.80-7.83 (d, *J*=7.6 Hz)	133.8
38	7.03-7.12(m)	115.0	6.83-6.85 (d, *J*=8.8 Hz)	116.5	7.11-7.19 (m)	115.0
39	-	147.3	-	148.3	-	147.4
40	-	129.5	-	129.2	-	129.5
41	8.55-8.62 (m)	127.7	8.35-8.40 (m)	127.2	8.58-8.64(m)	127.8
42	8.55-8.62 (m)	-	8.35-8.40 (m)	-	8.58-8.64 (m)	-
43	3.24-3.34 (m)	47.9	3.22-3.31 (m)	47.8	3.24-3.33 (m)	47.9
44	1.89 (m)	33.8	1.85-1.90 (m)	33.8	1.87-1.92 (m)	33.8
45	1.60-1.63 (m)	30.2	1.59-1.62 m)	30.2	1.60-1.63 (m)	30.1
46	3.83-3.86(m)	66.6	3.82-3.86 (m)	66.6	3.84-3.89 (m)	66.6
47	3.24-3.34 (m)	66.6	3.22-3.31 (m)	66.6	3.24-3.33(m)	66.6
48	1.23-1.29 (m)	30.2	1.17-1.29 (m)	30.2	1.21-1.28 (m)	30.1

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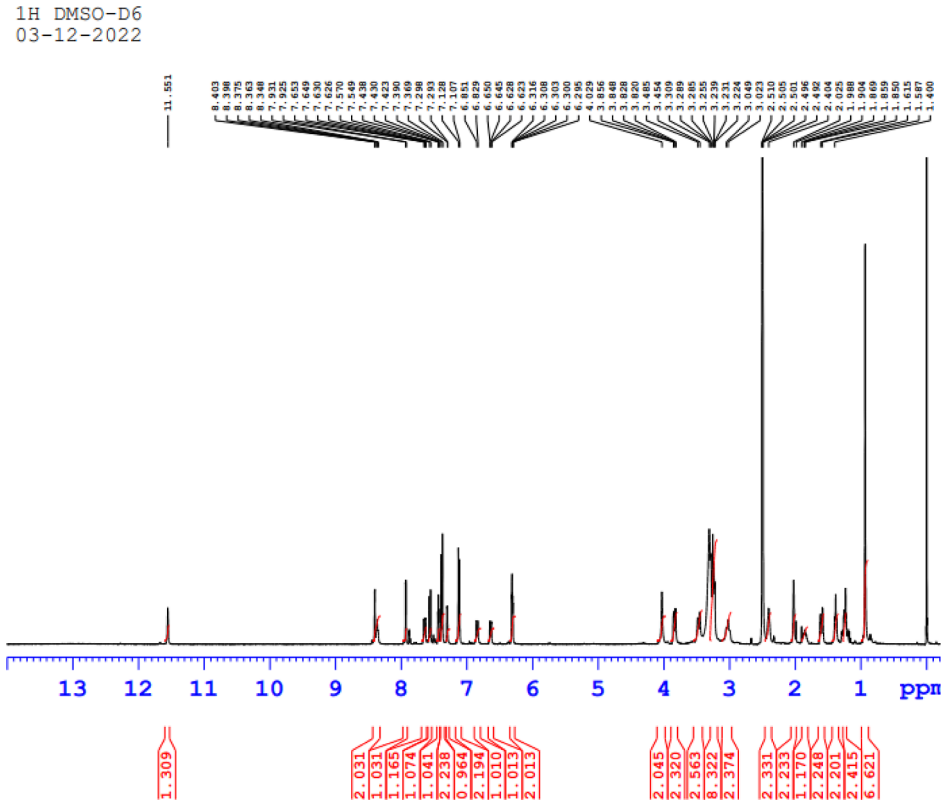

Figure 7. ^{1}H NMR spectrum of the venetoclax *N*-oxide impurity in DMSO-d_6.

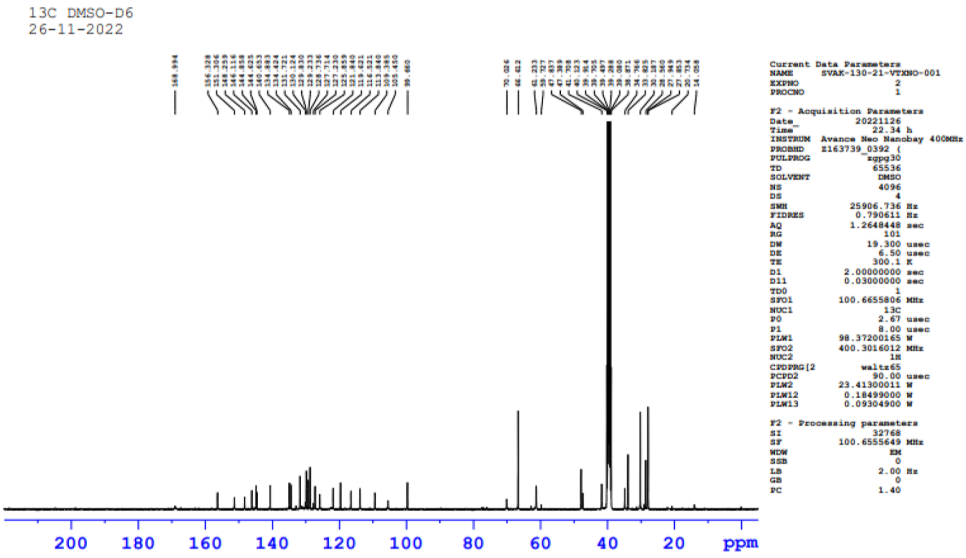

Figure 8. ^{13}C NMR spectrum of the venetoclax *N*-oxide impurity in DMSO-d_6.

Oxidative stress degradation was carried out by using 0.3, 1, 3, 5, and 10% H_2O_2 solutions.[19,20] After screening, the maximum percentage of the impurity was obtained by conducting oxidative stress using a 10% H_2O_2 solution. Venetoclax was dissolved in dichloromethane (DCM) (10 vol) to get a clear solution. These different concentrations of the H_2O_2 solution were added in different vials and kept under stirring for 4 h at room temperature (35−40 °C) in a dark place. The degradation was very slow in a 1 and 3% H_2O_2 solutions when compared with that in 5 and 10% H_2O_2 solutions. The results are given in the Results and Discussion section.

2.6. Synthesis of the VNO Impurity. To a stirred solution of venetoclax (3.0 g, 3.45 mmol) in DCM (30.0 mL, 10.0 vol) was added *m*-CPBA (0.89 g, 5.17 mmol) slowly at 10−15 °C for 30 min. The reaction was stirred for 30 min at the same temperature. After the completion of the reaction [checked using thin-layer chromatography (TLC)], aqueous $NaHCO_3$ solution (30.0 mL, 10.0 vol) was added at room temperature. The two layers were separated, and the aqueous layer was washed with DCM (20 mL). The organic layer was dried over anhydrous sodium sulfate. The organic solvent was evaporated under vacuum to get the crude compound, which is

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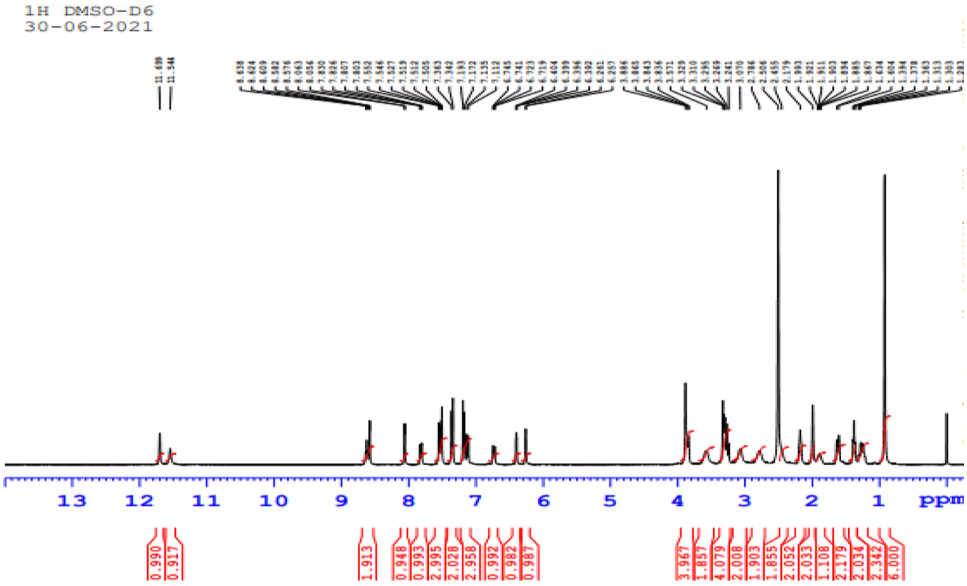

Figure 9. ^{1}H NMR spectrum of the VHA impurity in DMSO-d_6.

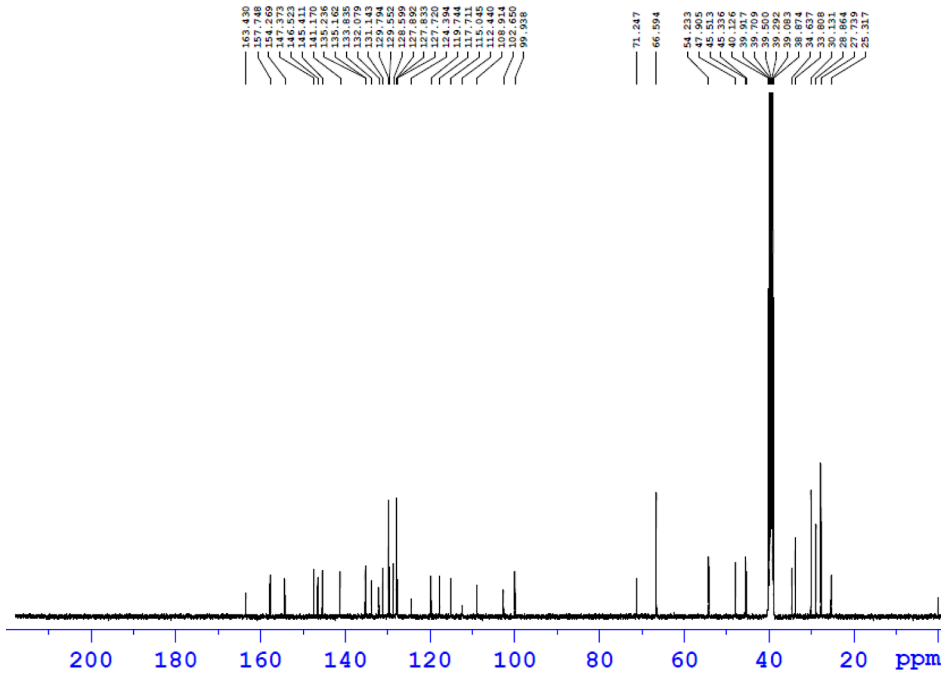

Figure 10. ^{13}C NMR spectrum of the VHA impurity in DMSO-d_6.

subjected to column purification using 10% MeOH in ethyl acetate to afford 1.84 g of a pale-yellow solid (Scheme 1). The pure compound was characterized by using different analytical techniques.

2.7. Synthesis of the VHA Impurity. Venetoclax *N*-oxide (0.50 g, 0.565 mmol) was taken in water (2.5 mL, 5 vol) in a 25.0 mL sealed tube. The reaction mixture was heated to 100−110 °C for 36 h. The reaction was monitored by TLC. After the completion of the VNO reaction, the product was purified by column chromatography using 1% MeOH in DCM to afford a yellow solid, which was analyzed by using NMR and mass spectroscopy analysis.

3. RESULTS AND DISCUSSION

An oxidative degradation study of venetoclax was performed using different concentrations of the H_2O_2 solution, i.e., 0.3, 1, 3, 5, and 10%. Venetoclax was dissolved in DCM to get a clear solution, followed by the addition of a 0.3% H_2O_2 solution to get only 1% of the VNO impurity after 24 h at room temperature, and we did not find any VHA impurity. When we facilitated the degradation by increasing the concentration of the H_2O_2 solution up to 10%, both the required impurities were formed at levels up to 6−8% after 36 h. The maximum impurity formation was observed in a 10% H_2O_2 solution. The impurity formation was analyzed by TLC (Figure 3).

During this stress degradation, we observed two major impurities via HPLC. To identify these two impurities, we

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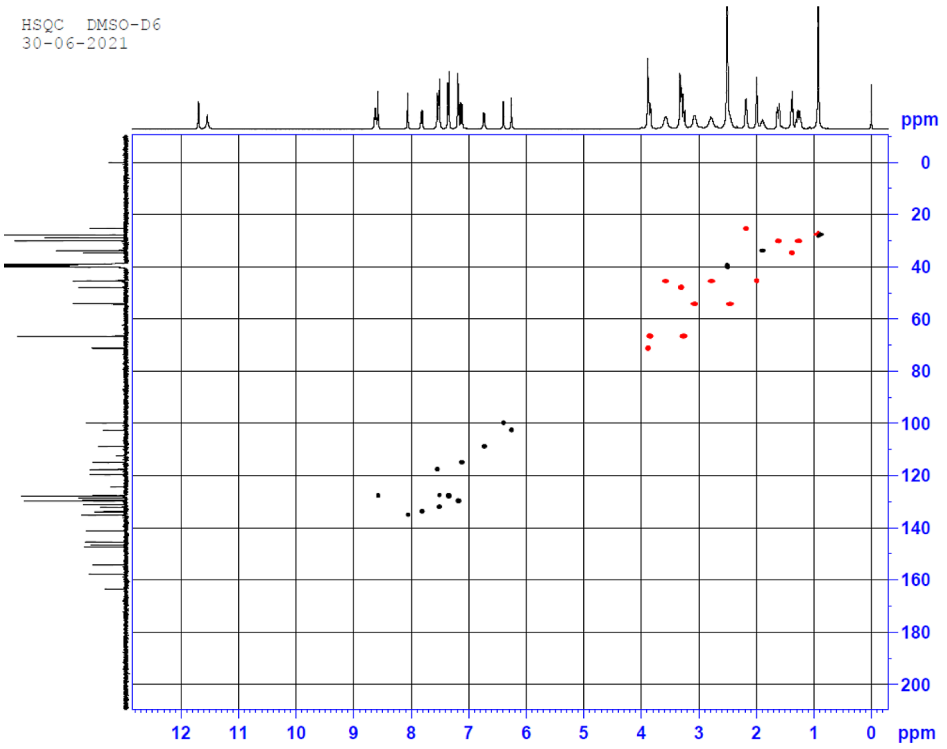

Figure 11. HSQC spectrum of the VHA impurity in DMSO-d_6.

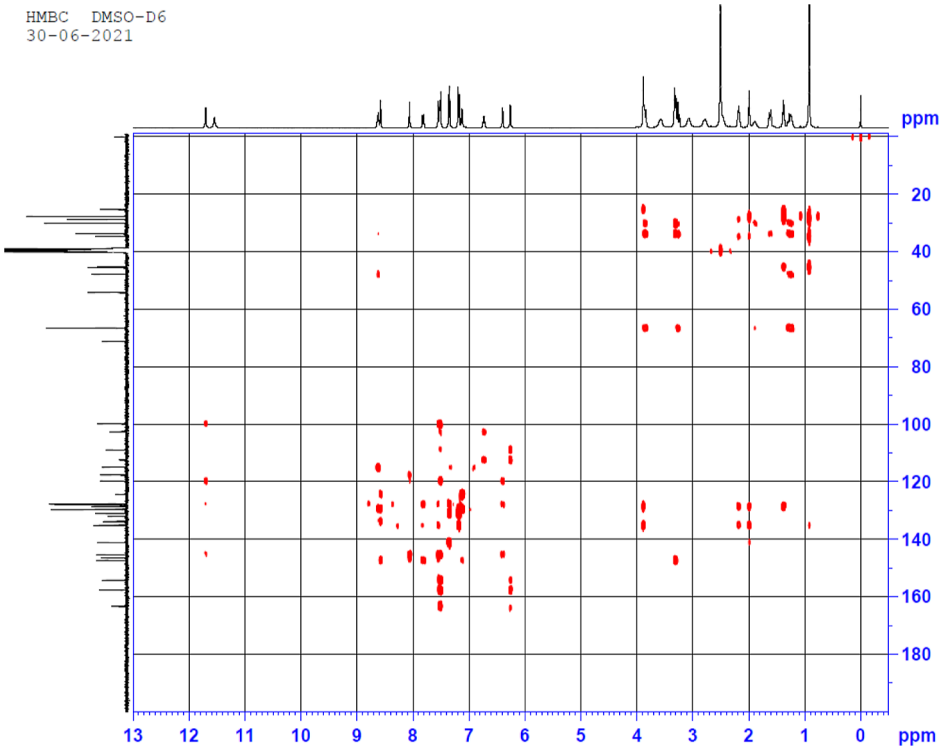

Figure 12. HMBC spectrum of the VHA impurity in DMSO-d_6.

subjected the degradation sample to LC−MS to find the molecular weight of the compounds. Surprisingly, both peaks showed the same molecular weight (884.27 in +ve mode) with different polarities. One peak is observed at 0.64 RRT, and another peak is observed at 1.13 RRT (Figure S15). To confirm these two compounds, we synthesized each impurity individually.

3.1. Identification/Detection of Unknown Impurities.

To identify the two impurities that are formed during the oxidative stress conditions of venetoclax, we performed LC−MS/MS analysis of the stressed sample. We obtained 884.36 in the positive mode and 882.26 in the negative mode for the polar impurity at RT 6.37 min and 884.27 in the positive mode and 882.24 in the negative mode for the nonpolar impurity at

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Table 2. DEPT and HMBC Assignment of the VNO Impurity and VHA Impurity

carbon number	DEPT	HMBC	carbon number	DEPT	HMBC
1	C	H-2,3,5,6	25	CH_2	H-21,22
2	CH	H-3,5,6	26	C	H-27,31,33
3	CH	H-2,5,6	27	CH	H-30,31,33
4	C	H-2,6,12	28	C	H-27,29,30,31,33
5	CH	H-2,3,6	29		
6	CH	H-2,3,5	30	CH	H-29,31,33
7	C	H-2,3,5,6,9,10,12,13,14,15	31	CH	H-29,30,33
8	C	H-9,10,12,15	32	C	H-29,30,31,33
9	CH_2	H-10,13,14,15	33	CH	H-27
10	CH_2	H-9,12,13,14,15	34	C	H-22,25
11	C	H-9,10,12,13,14,15	35		
12	CH_2	H-10,13,14	36	C	H-38,41
13	CH_3	H-9,10,12,14	37	CH	H-38,41
14	CH_3	H-9,10,12,13	38	CH	H-41
15	CH_2		39	C	H-37,38,41,43
16	CH_2		40	C	H-38,41
17	CH_2	H-10,13,14	41	CH	H-37,38,42
18	CH_2	H-10,13,14	42		
19	CH_2		43	CH_2	H-42,48
20	C	H-22,25	44	CH	H-42,43,45,46,47,48
21	CH	H-22,25	45	CH_2	H-43,44,46,47,48
22	CH		46	CH_2	H-43,44,47,48
23	C	H-21,25	47	CH_2	H-43,44,46,48
24	C	H-25,33	48	CH_2	H-43,44,46,47

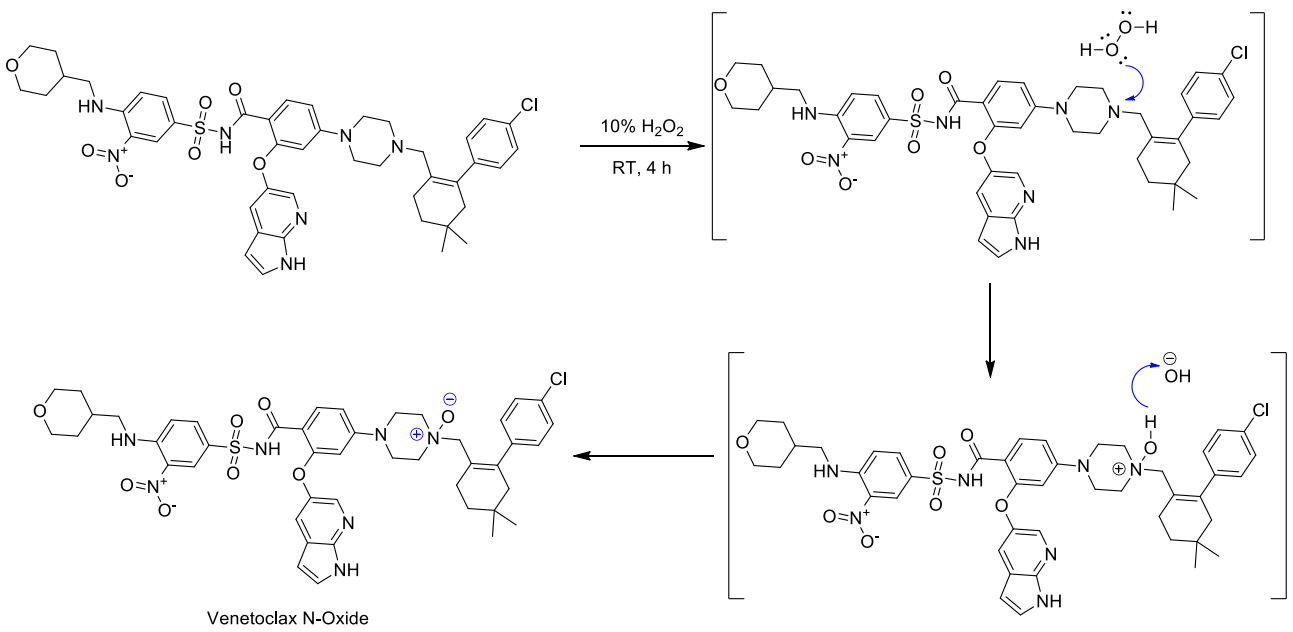

Figure 13. Plausible mechanism for the formation of the VNO impurity.

RT 12.48 (Figures 4–6). In TLC, we also observed the two impurities in a similar manner, i.e., one impurity at polar and another impurity at nonpolar when compared with venetoclax API.

Based on the mass spectrum, we expected the two impurities to have similar mass numbers due to the addition of oxygen on venetoclax. We predicted that venetoclax is forming *N*-oxide at the piperazine ring, which is the main reason to get the 884 mass value. But after seeing the HPLC and LC–MS spectra, we thought that there would be some change in the structure of the impurities due to the change in polarity observed in the chromatograms.

To confirm this, we have isolated these two compounds using preparative HPLC after the stress conditions. The two impurities were analyzed using several analytical methods, such as ^{1}H NMR, ^{13}C NMR, and mass spectrometry. For the VHA impurity, we have used 2D NMR [heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC)] to confirm the structure.

To confirm the venetoclax *N*-oxide (VNO) impurity, we reacted venetoclax with *m*-CPBA in DCM at 10–15 °C

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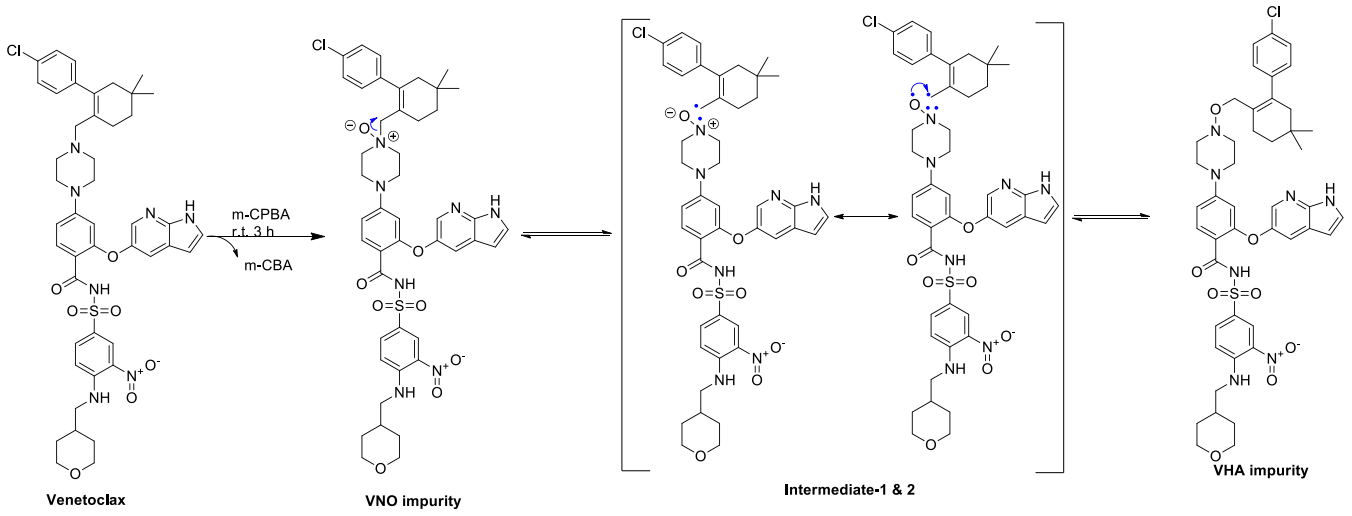

Figure 14. Plausible mechanism for the formation of the VHA impurity.

(Scheme 1). The reaction went smoothly to result in only one product, which matches with the more polar peak present in the HPLC chromatogram of the stressed sample. Further, to confirm this impurity, we analyzed this sample using ^{1}H NMR and ^{13}C NMR to confirm the structure of venetoclax *N*-oxide.

After isolating the VNO impurity, we have subjected it to mass analysis to get the molecular weight of the compound. The VNO impurity showed a peak at 884.37 in the positive mode and a peak at 882.26 in the negative mode.[20] Along with these, two fragment peaks were observed with mass numbers of 866.38 (loss of water) and 652.37 (loss of the chlorophenyldimethyl-cyclohexenyl moiety). The ^{1}H NMR and ^{13}C NMR values are presented in Table 1. In ^{1}H NMR spectra, we observed a change in the chemical shift values of three $-CH_2$ groups (H-15, H-16, and H-19) attached to the nitrogen of piperazine. Protons attached to H-15 were shifted from δ 2.75 to 4.03 ppm. H-16 and H-19 protons were shifted from δ 2.19 to 3.45−3.49 ppm, and H-16′ and H-19′ were shifted from δ 2.19 to 3.22−3.31 ppm. There was a slight change in the chemical shift of the methylene group (H-9) from δ 2.14 to 2.40 ppm (Figure 7).

Similarly, in the ^{13}C NMR spectrum (Figure 8), a change in the chemical shifts of methylene carbons attached to the piperazine ring was observed. The oxidation of the piperazine group of venetoclax to its corresponding *N*-oxide results in deshielding of the carbons attached to the nitrogen of the piperazine ring. Thus, *N*-oxide formation was caused by a down shield of methylene carbon at 15, 16, and 19 carbons, which were attached directly to the piperazine ring. The carbon attached to 15-CH_2 was shifted from δ 59.6 to 70.0 ppm, 16-CH_2 was shifted from δ 52.0 to 61.2 ppm, and 19-CH_2 was shifted from δ 52.0 to 59.7 ppm. We did not observe much change in the carbon value of the aromatic ring attached to the piperazine ring. After seeing all the values obtained from ^{1}H NMR and ^{13}C NMR spectra and mass spectra, it was confirmed that the *N*-oxide was formed on the piperazine nitrogen, which was attached to the aliphatic 15-methylene group.

To confirm the formation of the VHA impurity, we synthesized the VNO impurity and subjected it to a thermal rearrangement in a sealed tube using water as a solvent at reflux temperature. The reaction was kept for 36 h at the same temperature. About 10−15% of a nonpolar peak was observed by HPLC as well as by TLC when compared with venetoclax API. We isolated this compound using column chromatography and coinjected with the stressed sample. Both the peaks are observed at the same RT and having the same mass, i.e., 884. Then, we characterized the pure sample using ^{1}H NMR, ^{13}C NMR, and 2D NMR.

In the ^{1}H NMR spectrum (Figure 9), we observed a change in the chemical shift values of three methylene protons attached to the nitrogen of the piperazine ring (H-15, H-16, and H-19). Protons attached to H-15 ($-OCH_2$) were shifted from δ 2.75 to 3.84−3.89 ppm. H-16 and H-19 protons were shifted from δ 2.19 to 3.07 ppm, and H-16′ and H-19′ were shifted from δ 2.19 to 2.46 ppm. These chemical shift values were different when compared with those of the VNO impurity.

In the ^{13}C NMR (Figure 10) spectra of the VHA impurity, carbon at δ 71.2 corresponding to the $-CH_2$ group (15-CH_2) attached to the piperazine ring of veneclox was shifted downfield when compared with the chemical shift of Venetoclax (δ 59.6) and the VNO impurity (δ 70.0). This was due to the [1,2] Meisenheimer rearrangement of venetoclax *N*-oxide to form the VHA impurity. Similarly, the carbon present at 16-CH_2 and 19-CH_2, which were adjacent to the piperazine nitrogen, is shifted downfield from d 52.0 to 54.2. But in the VNO impurity, these two carbons are observed at d 61.2 and 59.7 due to the formation of *N*-oxide.

In the HSQC spectrum (Figure 11), it was confirmed that four $-CH_2$ protons (H-16, H-17, H-18, and H-19) of the piperazine ring were separated from one another when compared with *N*-oxide. Using HMBC spectra (Figure 12), it is evident that there is no correlation between H-15 with H-16 and H19, which shows the formation of the hydroxyalamine group in venetoclax.

By using all the above spectral data, it was confirmed that the above compound is a venetoclax hydroxylamine impurity (Table 2).

3.2. Plausible Formation Pathway of the VHA Impurity. Venetoclax *N*-oxide was formed due to the addition of oxygen to nitrogen of a piperazine ring by using hydrogen peroxide as an oxidative agent (Figure 13). The venetoclax hydroxyl amine impurity was obtained by the Meisenheimer rearrangement of venetoclax *N*-oxide. VNO undergoes classic Meisenheimer rearrangement by homolytic dissociation to

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form diradical intermediates (intermediate-1 and -2), in which the oxygen atom and methylene group remain on the same side. Due to this, the oxygen atom is unable to attack the double bond to form the [2,3]-Meisenheimer rearrangement product. It attacks from the same side to form a C−N bond to get the required VHA impurity by undergoing [1,2]-Meisenheimer rearrangement (Figure 14).

4. CONCLUSIONS

In conclusion, it was confirmed that the two impurities obtained from oxidative stress studies are 4-(3-((1*H*-pyrrolo-[2,3-*b*]pyridin-5-yl)oxy)-4-(((3-nitro-4-(((tetrahydro-2*H*-pyran-4-yl)methyl)amino)phenyl)sulfonyl)carbamoyl)-phenyl)-1-((4′-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)methyl)piperazine 1-oxide (venetoclax *N*-oxide, **VNO**) and 2-((1*H*-pyrrolo[2,3-*b*]pyridin-5-yl)oxy)-4-(4-((4′-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)-methoxy)piperazin-1-yl)-*N*-((3-nitro-4-(((tetrahydro-2*H*-pyran-4-yl)methyl)amino) phenyl)sulfonyl)benzamide (venetoclax hydroxylamine impurity, **VHA**). The two impurities were confirmed using ^{1}H NMR, ^{13}C NMR, and mass spectroscopy analysis. These two impurities were synthesized from the Venetoclax API. The formation of the VHA impurity was conveniently reported using Meisenheimer rearrangement. These impurities will be used as reference standards for researchers to identify the formation of oxidative impurities during the stability of venetoclax API and formulation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05325.

All the spectral data and LC−MS analysis of the synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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