



Synthesis, Isolation and Characterization of Process-Related Impurities in Oseltamivir Phosphate

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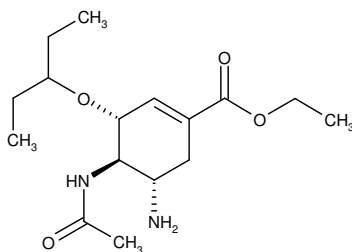
Abstract: Three known impurities in oseltamivir phosphate bulk drug at level 0.1% (ranging from 0.05-0.1%) were detected by gradient reverse phase high performance liquid chromatography. These impurities were preliminarily identified by the mass number of the impurities. Different experiments were conducted and finally the known impurities were synthesized and characterized.

Keywords: Oseltamivir phosphate, Impurities, Synthesis, Isolation and Characterization

Introduction

Oseltamivir is an ester prodrug, which is rapidly and extensively hydrolysed *in vivo* to its active metabolite oseltamivir carboxylate, a potent and selective inhibitor of influenza virus neuraminidase¹ is considered the leading currently available antiviral to counter a serious epidemic or pandemic outbreak of influenza^{2,3}. The current concerns over avian influenza A (H5N1) have created an increased demand for this drug. Pharmaceutical counterfeiting is a well recognized global health problem with a particular impact in developing countries where drug-regulatory systems are weak or ineffective⁴. There have been many alarming reports lasting recent years of counterfeit antimalarials, antibiotics, hormones and steroids, analgesics and antipyretics, anti-asthma and anti-allergy drugs⁵⁻¹¹.

Oseltamivir phosphate is a white crystalline solid with the chemical name (3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1). The chemical formula is C₁₆H₂₈N₂O₄ (free base). The molecular weight of oseltamivir is 312.4 and 410.4 for oseltamivir phosphate salt¹². The structural formula is as follows:



There are several process impurities/related substances associated with the manufacture of oseltamivir phosphate. Different process related impurities have been observed with various synthetic routes and/or manufacturing processes. Six of the known oseltamivir phosphate related substances have been mentioned in International Pharmacopoeia. All these impurities are never present together while we are preparing bulk drug or preparing formulation.

The International Pharmacopoeia¹² specifies that individual impurity is not more than 0.1% and total impurity is not more than 0.7%. During the process development of oseltamivir phosphate three known impurities were identified in the analysis of different batches whose percent area ranged from 0.05-0.1% by HPLC¹². A comprehensive study has been carried out to isolate or to prepare and characterize these impurities. To the best of our knowledge, synthesis route of impurities is novel and not reported in literature. Due to the importance of regulatory authorities¹³ all the impurities which are at the level of >0.1% must be identified and characterized.

A literature search revealed that only analytical procedure¹⁴⁻¹⁸ is available but nobody has reported synthesis, isolation and characterization of impurities in the purified form starting from oseltamivir phosphate. The present communication involves the isolation or preparation of impurities and characterization by chromatographic and spectroscopic techniques¹⁹.

Experimental

The investigated samples of bulk oseltamivir phosphate were received from pharماسolve specialities (I) Pvt. Ltd. Mumbai.

High performance liquid chromatography (HPLC)

An LC method was used for monitoring the reaction of oseltamivir impurities. A Shimadzu model LC2010CHT equipped with a UV detector was used. The chromatographic conditions optimized were ODS, 50×4.6 mm, 3 μ column with a mobile phase -A consisting 900:100(v/v) of 0.05 M KH₂PO₄ buffer pH 6.0 and acetonitrile and mobile phase-B consisting 100:900(v/v) of 0.05 M KH₂PO₄ buffer pH 6.0 and acetonitrile with a timed gradient programme of T/% B: 0/20, 3/50, 8/100, 15/100, 16/20, 20/20. Detection was carried out at 220 nm and the flow rate 1.5 mL/min. Data was recorded by using LC solutions software.

High performance liquid chromatography (preparative)

An inhouse LC method was developed for the isolation of oseltamivir impurities. A Waters LC controller equipped with a UV detector was used. The Prep-HPLC method uses a C18 column (YMC Pack Pro.C-18, 250 mm×20 mm i.d., 10 μm particle size) with a mobile phase A consisting of 0.02 M ammonium acetate and mobile phase B consisting acetonitrile timed gradient programme of T/% B: 0/0, 5/0, 8/80, 15/80, 16/0, 20/0. Detection was carried out at 207 nm and the flow rate was 15.0 mL/min. Data was recorded by using Empower software.

Mass spectrometry

Electrospray ionization mass spectrometry was performed using an ion trap mass spectrometer (Model 6310 agilent). The positive and negative electrospray MS data was obtained by switching the capillary voltage between $n+ 5000$ and $-4500V$ respectively.

NMR spectroscopy

The NMR experiments were performed on Bruker avance II 400 MHz. The 1H chemical shift values were reported on the δ scale in ppm, relative to TMS ($\delta=0.00$) and the ^{13}C chemical shift values were reported relative to $CDCl_3$ ($\delta=77.00$ ppm) and DMSO, d_6 ($\delta=39.50$ ppm) as internal standards. 1H and ^{13}C experiment was run using a mixing time of 1000 ns.

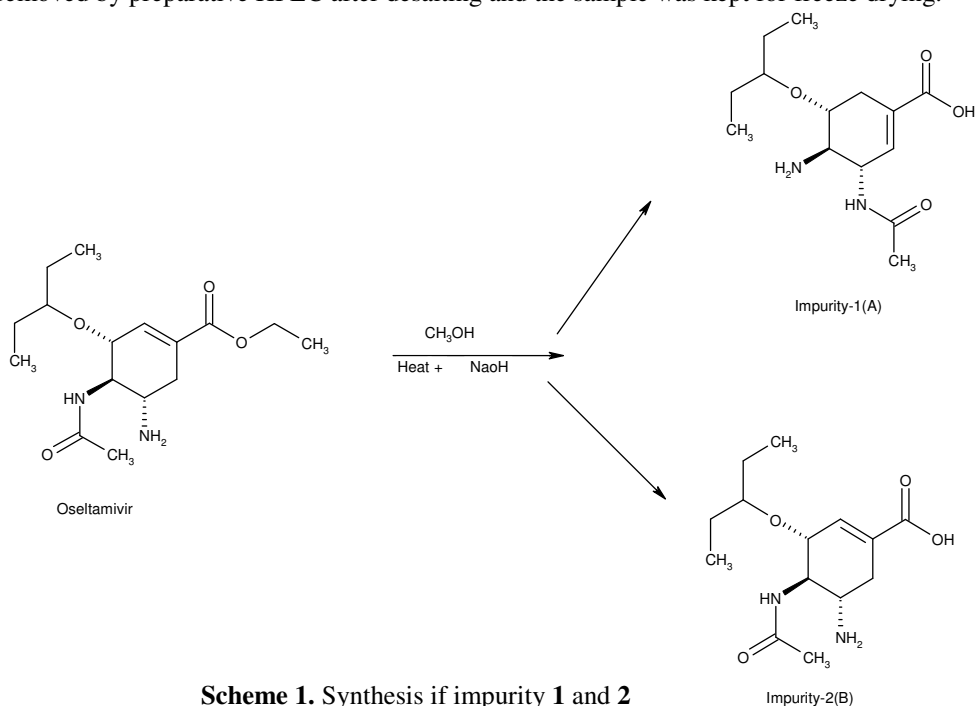
FT-IR spectroscopy

The IR spectra were recorded in the solid state as KBr dispersion medium using Perkin Elmer spectrum 100 FT-IR spectrophotometer.

Synthesis of impurities

Synthesis of impurity 1 and 2

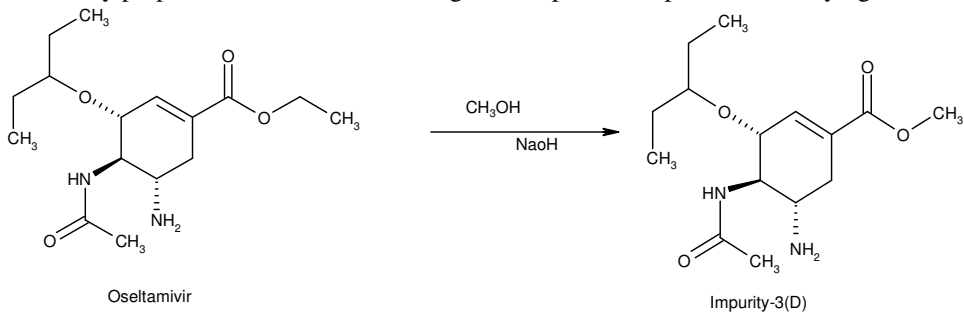
Oseltamivir base (1 g) was charged with 100 mL methanol into a double necked round bottom flask with magnetic stirrer. Methanolic sodium hydroxide was added drop wise and the reaction mass was heated very carefully and the reaction mass was refluxed at methanol boiling point. The reaction was monitored after every 1 h by HPLC. After completion of reaction the reaction mass was neutralised with dilute HCl. The HPLC analysis shown 2 peaks separately which confirm the presence of two different impurities. Both peaks were purified using preparative HPLC and marked as fraction-1 and 2 (Scheme 1). The salt was removed by preparative HPLC after desalting and the sample was kept for freeze drying.



Scheme 1. Synthesis of impurity 1 and 2

Synthesis of impurity 3

Oseltamivir base (1 g) was charged with 100 mL methanol into a double necked round bottom flask with magnetic stirrer. To this methanolic sodium hydroxide was added and the reaction mass was stirred at room temperature. The reaction was monitored after every 1 h by HPLC. The HPLC analysis had shown 1 peak which confirms the presence of one different impurity. This peak was purified using preparative HPLC and marked as fraction-3 (Scheme 2). After completion of reaction the reaction mass was neutralised with dilute HCl. The salt was removed by preparative HPLC after desalting the sample was kept for freeze drying.



Scheme 2. Synthesis of impurity 3

Results and Discussion

Structure elucidation of impurity 1 (Impurity-A)

Sample was analyzed by HPLC and its purity was found to be 94.13%, molecular weight of oseltamivir phosphate impurity-A is 284.36, 28 mass unit less than that of oseltamivir phosphate. The ESI mass spectrum of sample gave a protonated molecular ion at m/z 285.1, which is the same as oseltamivir phosphate impurity-A, IR spectrum displayed characteristic absorptions at 3430.01 & 2968.79, 2939.87 cm^{-1} corresponding to $>\text{NH}$ and aliphatic $>\text{CH}$ stretching. The peaks at 1642.69 & 1200.32 cm^{-1} in IR spectrum are indicative of $>\text{C}=\text{O}$ stretching & $\text{O}-\text{C}$ stretching, ^1H signals showed the absence of signals at 4.11-4.19 & 1.20-1.24 ppm was observed as multiplet & triplet, which could be attributed to an ethyl group absent in impurity-A, but a significant change was observed in the position of 1C & 5C protons which is due to a change in double bond position in impurity-A, ^{13}C signals showed absence of signals at 60.52 & 14.02 ppm, which could be attributed to an ethyl group absent in impurity-A, but a significant change was observed in the position of 1C & 5C carbons which is due to a change in double bond position in impurity-A. HPLC purity 94.13%, mass +ve m/z 285.1 (Table 1), IR (KBr): 3430.01, 2968.79, 2939.87, 1642.69 & 1200.32 (Table 1); ^1H NMR: 2.03-2.69 (dd, 2H), 3.30 (s, 1H), 3.54-3.56 (d, 1H), 3.95 (m*, 1H), 6.30 (s, 1H), 8.11 (s, 1H), 1.84 (s, 3H), 2.91 (s, 1H), 1.39 (s, 4H), 0.78-0.83 (d, 6H) (Scheme 3, Table 2); ^{13}C NMR: 32.72, 75.85, 55.55, 49.70, 134.94, 131.19, 169.97, 23.09, 80.58, 25.11, 25.74, 8.91, 9.46 (Scheme 4, Table 3).

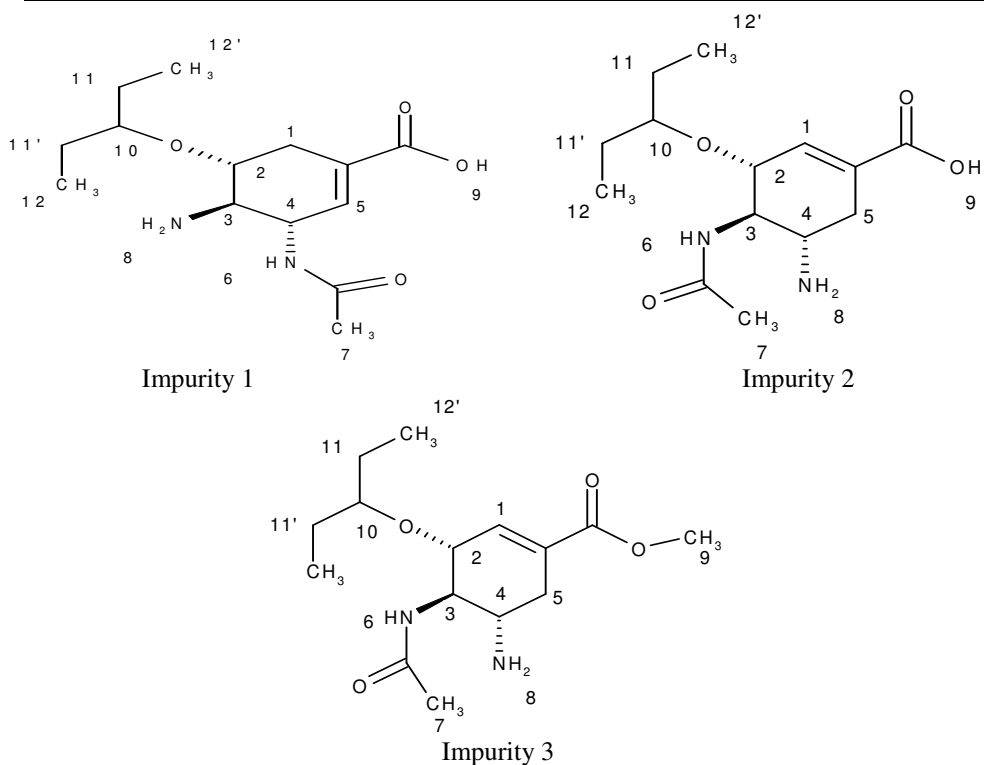
Structure elucidation of impurity 2 (Impurity-B)

Sample was analyzed by HPLC and its purity was found to be 92.61%, molecular weight of oseltamivir phosphate impurity-B is 284.36, 28 mass unit less than that of oseltamivir phosphate. The ESI mass spectrum of sample gave a protonated molecular ion at m/z 285.0, which is the same as oseltamivir phosphate impurity-B, IR spectrum displayed characteristic absorptions at 3419.69 & 2967.75, 2939.35 cm^{-1} corresponding to $>\text{NH}$ and aliphatic $>\text{CH}$

stretching. The peaks at 1646.68 & 1200.14 cm^{-1} in IR spectrum are indicative of $>\text{C}=\text{O}$ stretching & O-C stretching, ^1H signals showed the absence of signals at 4.11-4.19 & 1.20-1.24 ppm was observed as multiplet & triplet, which could be attributed to a ethyl group absent in Impurity-B, ^{13}C signals showed absence of signals at 60.52&14.02 ppm, which could be attributed to a ethyl group absent in Impurity-B. HPLC purity 92.61%, mass +ve m/z 285.0 (Table 1), IR (KBr): 3419.69, 2967.75,2939.35, 1646.68 & 1200.14 (Table 1); ^1H NMR: 6.19 (s, 1H), 3.38 (s, 1H), 3.83-3.85 (d,1H), 3.34-3.36 (m,1H), 2.53-2.60 (dd,2H), 1.83 (s, 3H), 3.26-3.29 (m,1H), 1.33-1.42 (m, 4H), 0.76-0.85 (m, 6H) (Scheme 3,Table 2); ^{13}C NMR: 137.41, 76.46, 57.97, 50.35, 35.38, 129.20, 169.49, 170.98, 22.99, 80.26, 25.19, 25.82, 8.97, 9.51 (Scheme 4, Table 3).

Table 1. IR and mass spectral data of impurities 1, 2 & 3

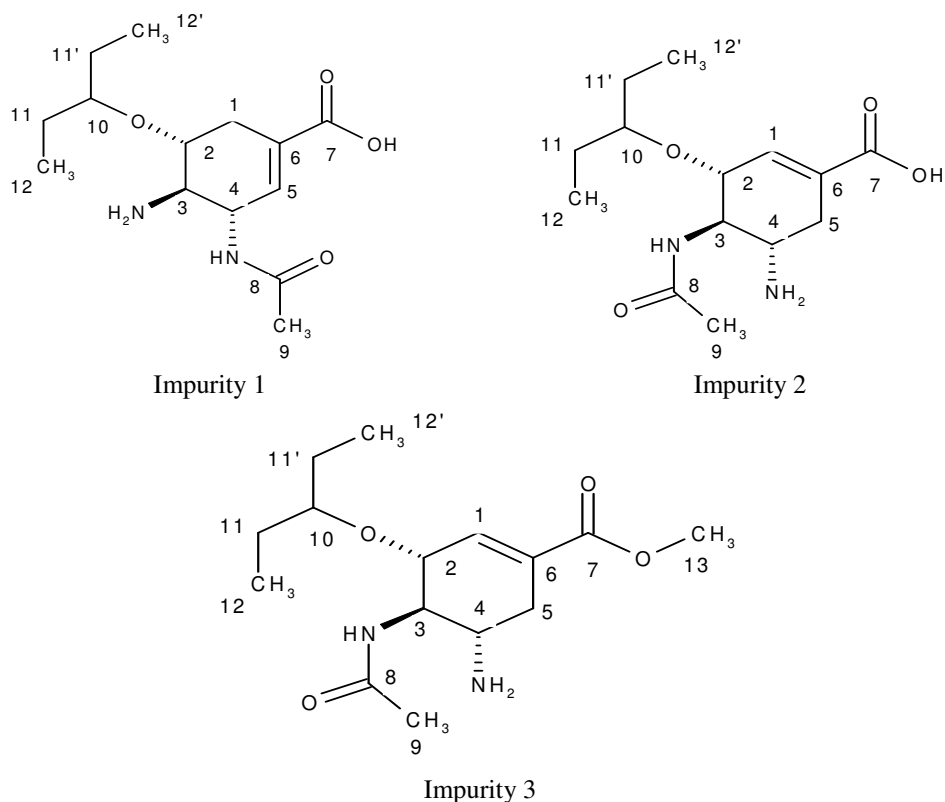
S.No.	Compound	IR cm^{-1}	MS
1	Impurity-1	3430.01 ($>\text{N-H}$) 2968.79,2939.87 (aliphatic $>\text{CH}$ stretching) 1642.69 & 1200.32 ($>\text{C}=\text{O}$ & O-C stretching) 3419.69 ($>\text{N-H}$)	+ve m/z 285.1
2	Impurity-2	2967.75,2939.35 (aliphatic $>\text{CH}$ stretching) 1646.68 & 1200.14 ($>\text{C}=\text{O}$ & O-C stretching) 3434.02 ($>\text{N-H}$)	+ve m/z 285.0
3	Impurity-3	2966.54,2939.46 (aliphatic $>\text{CH}$ stretching) 1648.06, 1249.80 ($>\text{C}=\text{O}$ & O-C stretching)	+ve m/z 299.1



Scheme 3. ^1H NMR assignment of impurities 1, 2 & 3

Structure elucidation of impurity 3 (Impurity-D)

Sample was analyzed by HPLC and its purity was found to be 97.37%, molecular weight of oseltamivir phosphate impurity-D is 298.39,14 mass unit less than that of oseltamivir phosphate. The ESI mass spectrum of sample gave a protonated molecular ion at m/z 299.1, which is the same as oseltamivir phosphate impurity-D, IR spectrum displayed characteristic absorptions at 3434.02 & 2966.54,2939.46 cm^{-1} corresponding to $>\text{NH}$ and aliphatic $>\text{CH}$ stretching. The peaks at 1648.06 & 1249.80 cm^{-1} in IR spectrum are indicative of $>\text{C}=\text{O}$ stretching & O-C stretching, ^1H signals showed the absence of signals at 4.11-4.19 & 1.20-1.24 ppm was observed as multiplet & triplet, which could be attributed to a ethyl group absent in impurity-D, but a singlet observed at 3.70 ppm which could be attributed to a methoxy group present in Impurity-D, ^{13}C signals showed absence of signals at 60.52 & 14.02 ppm, which could be attributed to a ethyl group absent in Impurity-D, there was a new ^{13}C signal at 48.95 ppm was observed which could be attributed to a methoxy group present in impurity-D. HPLC purity 97.37%, mass +ve m/z 299.1 (Table 1), IR (KBr): 3434.02, 2966.54,2939.46, 1648.06, 1249.80 (Table 1); ^1H NMR: 6.65 (s, 1H), 3.63-3.68 (m, 1H), 4.15-4.17 (m,1H), 3.37-3.40 (m,1H), 2.21-2.78 (dd, 2H), 8.16-8.18 (d, 1H), 1.87 (s, 3H), 5.49 (b, 2H), 3.70 (s, 3H), 3.09-3.16 (m,1H), 1.34-1.48 (m, 4H), 0.76-0.85 (m, 6H) (Scheme 3, Table 2); ^{13}C NMR: 138.60, 74.37, 53.74, 51.97, 29.87, 127.35, 170.28, 165.80, 23.19, 81.01, 24.97, 25.58, 8.88, 9.42, 48.95 (Scheme 4, Table 3).



Scheme 4. ^{13}C NMR assignment of impurities 1,2 & 3

Table 2. ¹H NMR spectral data of impurities **1, 2 & 3**

S.No.	Impurity 1		Impurity 2		Impurity 3	
	δ , ppm	H	δ , ppm	H	δ , ppm	H
1	2.03-2.69	2H	6.19	1H	6.65	1H
2	3.30	1H	3.38	1H	3.63-3.68	1H
3	3.54-3.56	1H	3.83-3.85	1H	4.15-4.17	1H
4	3.95	1H	3.34-3.36	1H	3.37-3.40	1H
5	6.30	1H	2.53-2.60	2H	2.21-2.78	2H
6	8.11	1H			8.16-8.18	1H
7	1.84	3H	1.83	3H	1.87	3H
8					5.49	2H
9					3.70	3H
10	2.91	1H	3.26-3.29	1H	3.09-3.16	1H
11,11'	1.39	4H	1.33-1.42	4H	1.34-1.48	4H
12,12'	0.78-0.83	6H	0.76-0.85	6H	0.76-0.85	6H

Table 3. ¹³C NMR spectral data of impurities **1, 2 & 3**

Carbon assignment	Impurity 1	Impurity 2 δ , in ppm	Impurity 3
1	32.72	137.41	138.60
2	75.85	76.46	74.37
3	55.55	57.97	53.74
4	49.70	50.35	51.97
5	134.94	35.38	29.87
6	131.19	129.20	127.35
7	169.97	169.49	170.28
8	169.97	170.98	165.80
9	23.09	22.99	23.19
10	80.58	80.26	81.01
1111'	25.1125.74	25.1925.82	24.9725.58
1212'	8.919.46	8.979.51	8.889.42
13			48.95

Conclusion

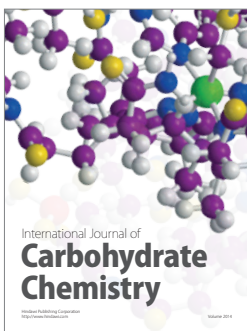
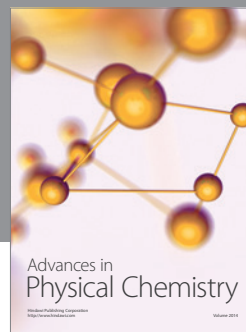
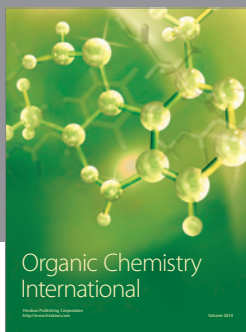
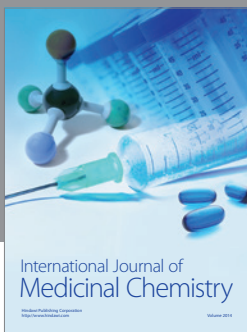
This research paper describes the synthesis, isolation and structure elucidation of process related impurities in oseltamivir phosphate. The impurities was separated by reverse phase chromatographic technique, further isolated these impurities by semi preparative liquid chromatography. The isolated impurities was characterized using spectroscopic techniques. This isolated impurities was used for mass balance of oseltamivir phosphate technical material. The synthesis of impurities was also discussed in brief.

Acknowledgment

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