Preparation of three impurities in cefoxitin

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Three known impurities of cefoxitin have been prepared and characterised. One is a double-bond isomer of cefoxitin, Δ^3-cefoxitin, which was easily prepared by base-catalysed isomerisation of cefoxitin. The second is a side-chain methoxylated derivative, methoxycefoxitin, which required a four-step synthesis from cephalothin. The last impurity is a lactone lacking the carbamyl grouping in cefoxitin, cefoxitin lactone, which was also prepared in four steps from cephalothin. The accessibility of these three impurities and methods for their analysis could be important for quality control in the manufacture of cefoxitin.

Keywords: cefoxitin impurity, Δ^3-cefoxitin, cefoxitin lactone, methoxycefoxitin

Cefoxitin (**1**, Fig. 1), a second-generation cephalosporin antibiotic, shows excellent efficacy due to its long-acting and broad-spectrum antibacterial properties as well as less potential nephrotoxicity and ototoxicity.[1,2] The mechanism of action of cefoxitin is similar to other second-generation cephalosporins. However, the methoxy group of cefoxitin significantly reduces the hydrolysis by β-lactamases in bacteria.[3] Therefore, the drug-resistance problem is much less serious than for other second-generation cephalosporins.

As treatment of bacterial infections requires a large dose (adult daily i.v. dose is 3 g), impurities related to the active pharmaceutical ingredient is a significant concern.[4] The structures of seven impurities for cefoxitin appeared in the *European Pharmacopoeia* 9th edition (2016).[5] Vundavilli and co-workers also reported an impurity in cefoxitin resulting from stress stability studies.[6] Owing to the poor stability of cephalosporins, the synthesis of impurities can be difficult. The structures and simplified names of three of the impurities, **2**, **3** and **4**, are shown in Fig 1, none of which are available commercially and no preparation method has been reported. Impurity **2** is a double-bond isomer of cefoxitin, impurity **3** is a side-chain methoxylated derivative and impurity **4** is a lactone derived by ring closure of the 10-hydroxymethyl derivative formed by cleavage of the carbamate residue of cefoxitin. According to the guidelines on impurities in drug substances suggested by the International Conference on Harmonisation,[7] it is necessary to have samples of these impurities in pure form for analytical method development. Herein, we report methods for the preparation of impurities **2**, **3** and **4** and their characterisation by ^{1}H NMR, ^{13}C NMR and 2D-NMR spectroscopy.

Results and discussion

Preparation and characterisation of impurity Δ^3-cefoxitin **2**

Rao and co-workers[8] reported that treatment of natural Δ^3-cephalosporin derivatives with triethylamine in methanol converted them into an equilibrium mixture of the natural Δ^3-cephalosporin derivatives and the corresponding Δ^2-cephalosporin derivatives. Using this methodology,[8] we found that cefoxitin **1** was isomerised at room temperature to an equilibrium mixture of **1** and Δ^3-cefoxitin **2** (Scheme 1). High performance liquid chromatography (HPLC) analysis was used to establish the ratio of **1** and **2** as 8:1 (Fig. 2), and preparative HPLC of the mixture permitted the isolation of a pure sample of **2**. The HRMS spectrum of impurity **2** showed a protonated molecular peak at 450.0404 $[M + Na]^+$ in positive-ion mode, indicating the mass of this impurity to be 427.04, which is the same mass as **1**. The isolated impurity **2** and cefoxitin **1** were subjected to 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (HSQC and HMBC) analysis. As an isomer of **1**, impurity **2** has many closely comparable chemical shift values, but some are different (see Table 1 for ^{1}H and ^{13}C assignments of **1** and **2**). As expected, the most dramatic changes affect carbons 9, 10 and 11 and the attached protons. In the ^{1}H NMR spectrum of the isomer **2**, the double-bond proton at H11 and the vinylic proton at H9 were observed at δ 6.25 (1H) and 4.80 (1H) ppm, respectively, whereas in cefoxitin **1** no proton was observed at C9 and H11 showed two geminally coupled signals (J = 18 ppm) at δ 3.55 and 3.21 ppm due to a methylene group. In the ^{13}C NMR

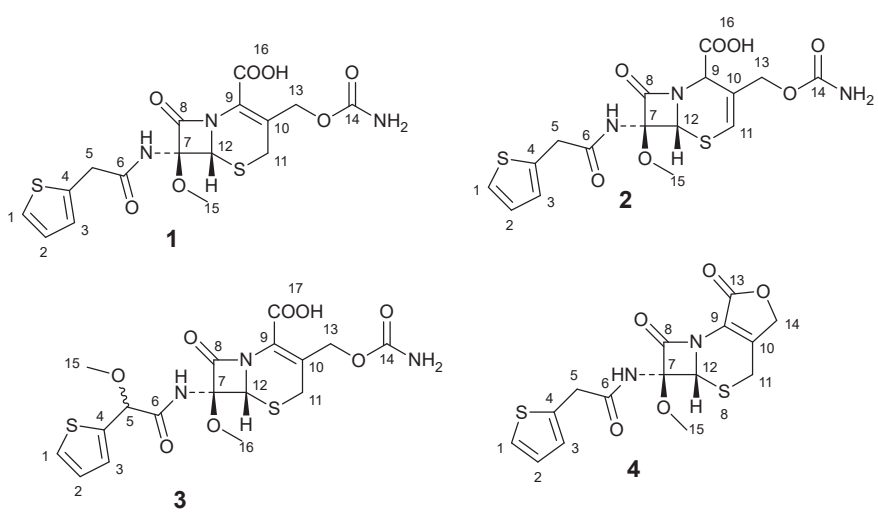

Fig. 1 Chemical structures of cefoxitin **1** and three impurities, Δ^3-cefoxitin **2**, (*R*,*S*)-5-methoxycefoxitin **3** and cefoxitin lactone **4**.

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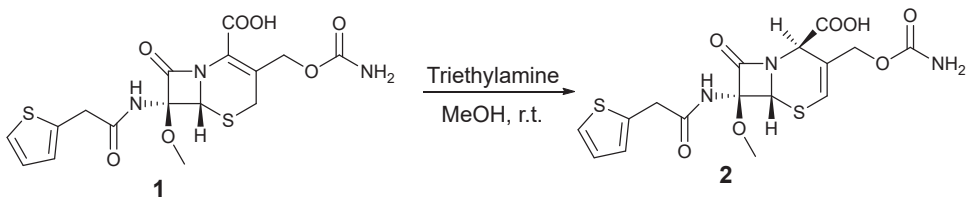
spectrum of isomer **2**, the C11 and C9 signals were observed at δ 120.5 and 54.9 ppm due, respectively, to sp^2 and sp^3 carbons in contrast to those of cefoxitin **1**, which appeared at δ 25.2 and 115.3 ppm, due, respectively, to sp^3 and sp^2 carbons. The above data and the results of 1D NMR and 2D NMR experiments confirmed the structure of impurity **2**.

Preparation and characterisation of impurity **3**

During optimisation of the synthesis of the 7-methoxylate-dintermediate **6** from the known compound, cephalothin **5**, using a reported method,[9] it was found that a by-product **7** was easily generated (Scheme 2). As the amount of *t*-butyl hypochlorite[10] was increased, the yield of by-product **7** increased simultaneously. The molecular weight of **7** was determined by liquid chromatography–mass spectrometry (LC-MS), which displayed a protonated molecular ion peak at *m/z* 474 $[M + NH_4]^+$ in positive-ion mode, indicating the mass of this by-product to be 456, which is 30 amu more than **6**. The extra 30 amu is equivalent to a methoxy group. The exact structure of by-product **7** was



Scheme 1

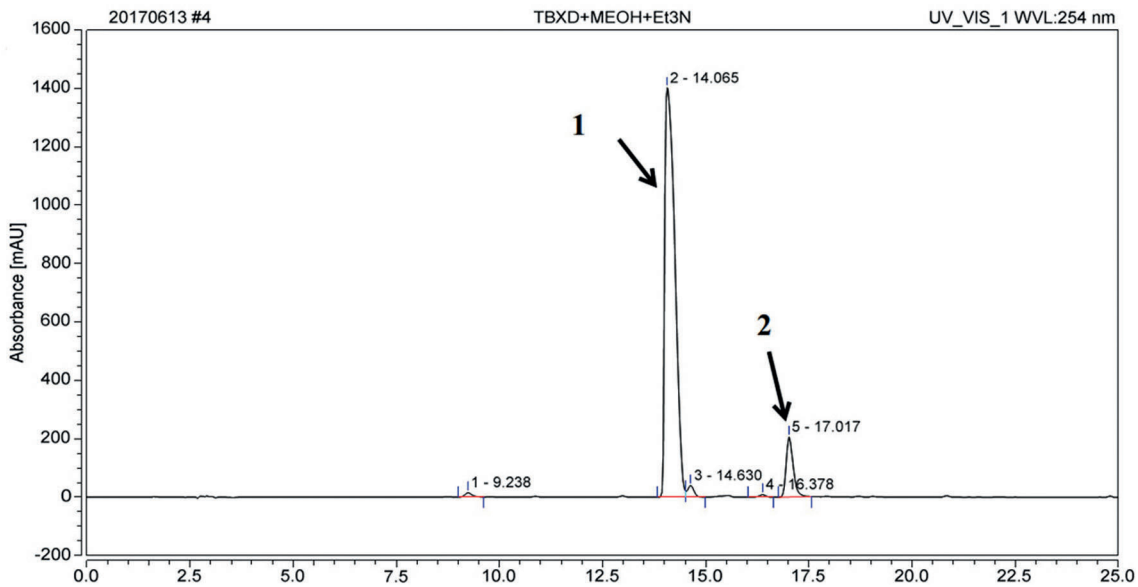
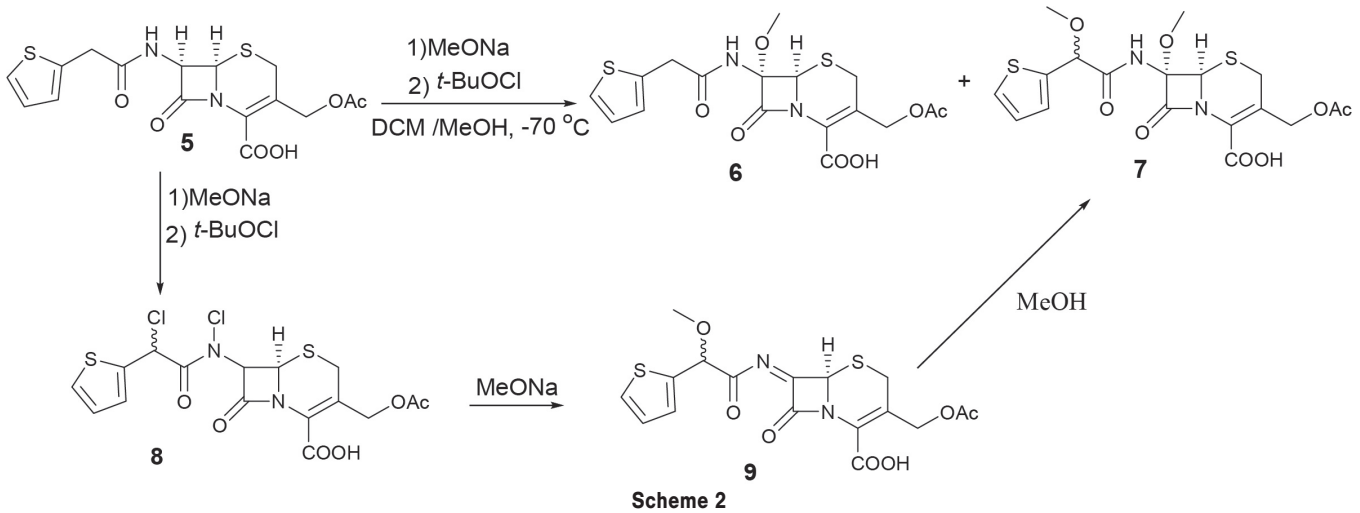


Fig. 2 A typical trace of a HPLC run (for eluting conditions, see Experimental), which shows the separation of cefoxitin **1** and isomeric impurity **2**.

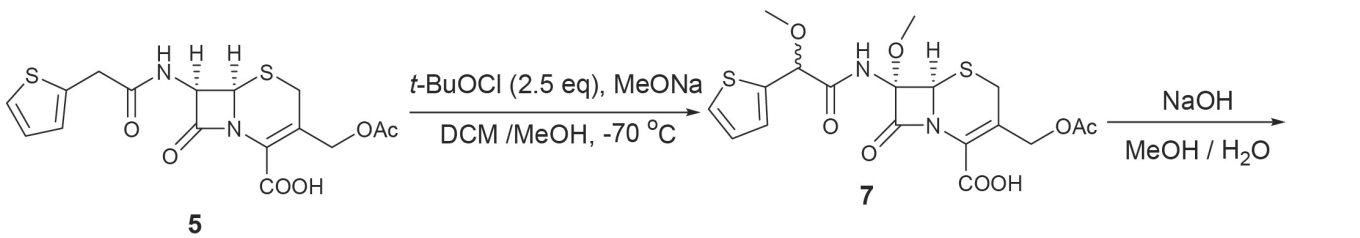
Table 1 ^1H and ^{13}C NMR data and the results of DEPT experiments for **1** and impurity **2**

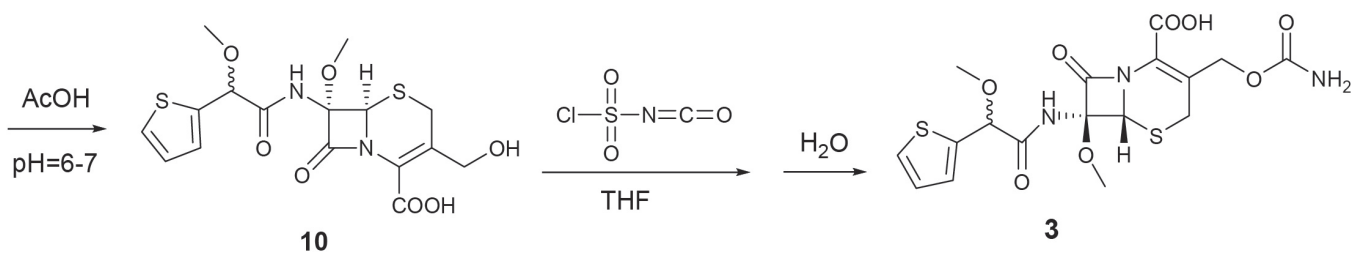
Position[a]	1				2			
	^1H	*J* (Hz)	^{13}C	DEPT	^1H	*J* (Hz)	^{13}C	DEPT
1	7.30	dd, J_1 = 1.2 Hz, J_2 = 5.4 Hz, 1H	124.5	CH	7.29	dd, J_1 = 1.2 Hz, J_2 = 5.4 Hz, 1H	126.5	CH
2	6.97	dd, J_1 = 3.6 Hz, J_2 = 5.4 Hz, 1H	126.3, 126.5	CH	6.97	dd, J_1 = 3.6 Hz, J_2 = 5.4 Hz, 1H	128.3, 128.5	CH
3	7.02	m, 1H	–	–	7.01	m, 1H	–	–
4	–	–	135.9	–	–	–	137.5	–
5	3.86	dd, J_1 = 15.0 Hz, J_2 = 33.0 Hz, 2H	–	CH_2	3.91	dd, J_1 = 15.6 Hz, J_2 = 44.4 Hz, 2H	38.0	CH_2
6	–	–	172.6	–	–	–	174.7	–
7	–	–	95.1	–	–	–	97.4	–
8	–	–	159.8	–	–	–	162.6	–
9	–	–	115.3	–	4.80	d, *J* = 1.2 Hz, 1H	54.9	CH
10	–	–	132.5	–	–	–	124.3	–
11	3.55 3.21	d, *J* = 18.0 Hz, 1H d, *J* = 17.4 Hz, 1H	25.2	CH_2	6.25	d, *J* = 1.2 Hz, 1H	120.5	CH
12	5.05	s, 1H	63.4	CH	5.48	d, *J* = 1.2 Hz, 1H	61.5	–
13	4.85	d, *J* = 12.0 Hz, 1H	63.8	CH_2	4.77	d, *J* = 12.0 Hz, 1H	67.9	CH_2
13	4.77	d, *J* = 12.0 Hz, 1H	63.8	CH_2	4.62	d, *J* = 12.6 Hz, 1H	67.9	CH_2
14	–	–	158.4	–	–	–	160.0	–
15	3.49	s, 3H	52.1	CH_3	3.49	s, 1H	54.3	CH_3
16	–	–	167.4	–	–	–	174.0	–

[a]s, singlet; d, doublet; dd, doublet of doublet; m, multiplet; for numbering, see chemical structure in Fig. 1.



Scheme 2





Scheme 3

characterised by ^{1}H NMR and ^{13}C NMR, confirming that the introduced methoxy group was located at C5.

According to literature,[11–13] a possible mechanism for the formation of by-product **7** was proposed (Scheme 2). Intermediate **5** undergoes N-chlorination of the amide bond and chlorination of the thienobenzyl side chain by *t*-butyl hypochlorite to generate **8**, which suffers elimination of HCl from the chloroamide and nucleophilic substitution by MeO^- of the side-chain Cl group to give the acylimine intermediate **9**. The strongly electrophilic acylimine **9** readily adds methanol to afford by-product **7**. By-product **7** was a key intermediate in the synthesis of impurity **3** described below.

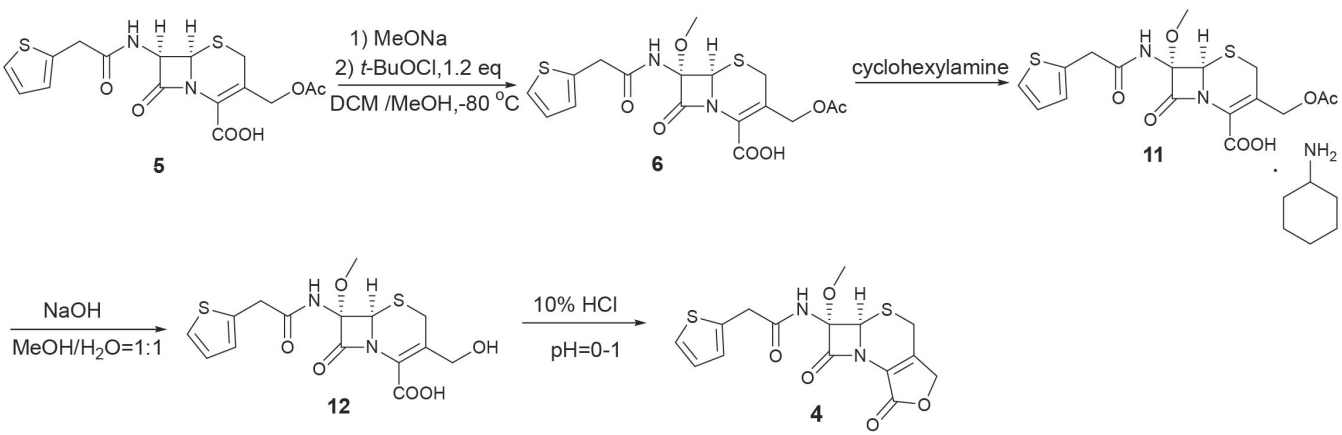
Based on the reported synthesis route of cefoxitin (**1**),[9] impurity **3** was prepared in four steps starting from cephalothin (**5**) (Scheme 3). Compound **5** was methoxylated with 2.5 equiv. of *t*-butyl hypochlorite and 6 equiv. of sodium methoxide at −75 °C to give **7**. Intermediate **7** was treated with sodium hydroxide in a mixture of methanol and water at −30 °C to afford hydroxymethylated derivative **10**. The intermediate **10** is extremely unstable under acidic conditions, readily cyclising to a lactone. We tried to apply the cefoxitin synthetic procedure of salt precipitation[9], but failed. After the base hydrolysis was completed, the reaction mixture was adjusted to pH 6–7 by acetic acid to prevent the formation of lactone. Removal of methanol and water under reduced pressure was followed by a solvent switch to tetrahydrofuran. Then chlorosulfonyl isocyanate underwent a nucleophilic addition with a slurry of intermediate **10** in tetrahydrofuran at −40 °C to yield the corresponding *N*-chlorosulfonyl carbamate, further hydrolysis of which gave crude **3**. Crude **3** was subjected to semi-preparative liquid chromatography and the fractions collected were lyophilised to remove the solvent to obtain **3** with a purity of 96%.

The high-resolution mass spectrum of **3** showed a molecular ion peak at *m/z* 480.0511 $[M + Na]^+$ in positive-ion mode, indicating the mass of **3** to be 457.05, which is 30 amu more than cefoxitin (**1**). The characterisation of impurity **3** was achieved by 1D NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D NMR (HSQC and HMBC) analysis (see Table 2 for ^{1}H and ^{13}C assignments). Compared with cefoxitin (**1**), impurity **3** showed one more methoxy group (–OCH_3). In the HMBC spectrum, the C15 protons at δ 3.47 ppm are coupled with C5 (δ 79.3 and 78.7 ppm), since impurity **3** is a pair of diastereomers and H5 appears as two signals (δ 5.14 and 5.10 ppm). The protons at δ 3.52 and 3.43 ppm are coupled with C7 (δ 95.7 and 95.6 ppm) and the C5 protons at δ 5.14 and 5.10 ppm are coupled with C1, C2, C3, C4 and C6 and C15, respectively. The C13 protons at δ 5.05 ppm are coupled with C7, C8 and C11. In the ^{1}H NMR spectrum, the protons of the thiophene ring appeared at δ 7.45–7.03 ppm (3H). In the DEPT spectrum of **3**, the negative-ion peaks of methylene carbons at δ 62.7 and 25.7 ppm correlated

Table 2 ^{1}H and ^{13}C NMR data and the results of COSY, DEPT, HSQC and HMBC experiments for impurity **3**

Position[a]	^{1}H	*J* (Hz)	COSY	^{13}C	DEPT	HMBC	HSQC
1	7.45	m, 1H	H2	126.6, 126.4	CH	C2, C3, C4	126.6, 126.4
2	7.03	m, 1H	H1, H3	126.3, 126.2	CH	C1, C3, C4	126.3, 126.2
3	7.24	m, 1H	H2	127.3, 127.1	CH	C1, C2, C4, C5	127.3, 127.1
4	–	–	–	139.0	–	–	–
5	5.14, 5.10	s, 1H	–	79.3, 78.7	CH	C1, C2, C3, C4, C6, C15	79.3, 78.7
6	–	–	–	172.0	–	–	–
7	–	–	–	95.7, 95.6	–	–	
8	–	–	–	160.5	–	–	–
9	–	–	–	128.9, 128.5	–	–	–
10	–	–	–	123.5	–	–	–
11	3.59–3.55	m, 1H	–	25.7	CH_2	C9, C10, C12, C13	25.7
11	3.37–3.30	m, 1H	–	25.7	CH_2	C9, C10, C12, C13	25.7
12	5.08	d, J = 1.2 Hz, 1H	–	64.2	CH	C7, C8, C11	64.2
13	5.05	dd, J_1 = 2.4 Hz, J_2 = 13.8 Hz,1H	–	62.7	CH_2	C9, C10, C11, C14	62.7
13	4.84	d, J_1 = 4.2 Hz, J_2 = 13.2 Hz, 1H	–	62.7	CH_2	C9, C10, C11, C14	62.7
14	–	–	–	158.0	–	–	–
15	3.47	s, 3H	–	56.5, 56.4	CH_3	C5	56.5, 56.4
16	3.46, 3.43	s, 3H	–	52.6, 52.5	CH_3	C7	52.6. 52.5
17	–	–	–	163.6	–	–	–

[a]s, singlet; d, doublet; dd, doublet of doublet; m, multiplet; for numbering, see chemical structure in Fig. 1.



Scheme 4

with C13 and C11, respectively. The above data and the 1D NMR and 2D NMR experiments confirmed the structure of **3**. Since impurity **3** was a pair of diastereomers, HPLC analysis showed two peaks.

Preparation and characterisation of impurity **4**

During our studies of the hydrolysis of **11** to **12** by a reported method,[9] we found that the hydroxymethyl acid **12** was easily converted to lactone impurity **4** at pH 0–2. Therefore, we carried out the hydrolysis of **12** in a mixture of methanol and 10% HCl at room temperature and pH 0–2 for 5 h to transform **12** into impurity **4** completely (Scheme 4). To obtain high purity **4**, the precursor of **12**, the methoxylated intermediate **6** was purified by formation of its cyclohexylamine salt **11**, which was isolated as a pale yellow solid. The crude **4** was purified by column chromatography to yield a pale yellow solid (m.p. 161–162 °C). The HRMS spectrum of **4** exhibited a molecular ion peak at *m/z* 389.0240 $[M + Na]^+$ in positive-ion mode, indicating the mass of **4** to be 366. ^{1}H NMR and ^{13}C NMR analyses revealed that the impurity **4** had one amide N–H proton (s, 9.54 ppm, 1H) and three well-resolved carbonyl carbon signals were observed at δ 170.1, 166.5 and 159.6 ppm. The proton signals of the thiophene ring appeared at δ 7.38 (1H) and 6.97–6.94 (2H) ppm, and the carbon signals at δ 125.6, 126.9 and 127.1 ppm, which are characteristic of thiophene carbon signals. In the DEPT spectrum of **4**, the negative-ion peaks of methylene carbons at δ 71.9, 36.3 and 23.3 ppm were assigned, respectively, to C14, C5 and C11. In addition, the FTIR spectrum of impurity **4** exhibited three strong characteristic absorption bands at ν = 1789, 1751 and 1666 cm^{-1} indicating the presence of three carbonyl groups.

To determine ways of improving the synthetic pathway of an active pharmaceutical ingredient, it is necessary to identify the impurities formed. The preparation of three impurities of cefoxitin described in this paper has never been reported. In addition, these impurities were characterised by NMR and HRMS spectral data. This work will be useful for quality control in the manufacture of cefoxitin.

Experimental

Chemical, reagents and samples

Cefalothin (**5**) and cefoxitin (**1**) were procured from Hubei Jusheng Technology Co., Ltd. Analytical reagents formic acid, acetic acid, ammonium acetate and HPLC grade acetonitrile and ethanol were procured from Shanghai Titan Technology Co., Ltd. Conventional solvents and reagents were purchased from Sinopharm Chemical Reagent Co., Ltd.

High-performance liquid chromatography

All reactions were monitored by HPLC on a Dionex Ultimate 3000 HPLC instrument using Shiseido ACR C18 (5 μm, 4.6 mm × 250 mm). The

mobile phase consisting of A (100 mmol L^{-1} NH_4OAc, pH 5.9, adjusted by acetic acid) and B (acetonitrile:ethanol = 15:7) was used in the gradient mode at a flow rate of 1.0 mL min^{-1}. The column was thermostated at a temperature of 40 °C and UV detection at 254 nm was used. The initial gradient was 10% of mobile phase B and at 10 min it was set to 15%. The ratio was set to 20% at 15 min, and then 35% at 30 min, followed by the ratio of mobile phase B set to 10% at 40 min, which was continued for 50 min. HPLC purity was reported in area %.

Preparative liquid chromatography

Preparative HPLC separation and fraction collections were carried out using a Waters 2767-2454 preparative liquid chromatograph equipped with a 2996 PDA detector. The sample solutions (acetonitrile:water = 1:1), about 40 mg mL^{-1} concentration, were prepared in diluent. The column, a Waters Xbridge C18 with 150 mm × 19 mm, 5 µm particle size was used for chromatographic separation. A 0.1% aqueous solution of trifluoroacetic acid and acetonitrile were used as solvent A and solvent B, respectively, as the mobile phase. A flow rate of 15 mL min^{-1} was employed throughout the process and the wavelength of detection in UV was 254 nm. The linear gradient programmes were set as time in minutes (T_{min})/solvent A:solvent B $T_{0.01}$/90:10, T_{10}/40:60, T_{11}/05:95 and T_{15}/05:95 for the isolation of impurities **2** and **3**. The crude sample was dissolved in 50% acetonitrile aqueous solution (concentration of 40 mg mL^{-1}). The solution was loaded into the preparative liquid chromatograph using the conditions mentioned in the above analytical liquid chromatography. Fractions were collected through repeated injections. The collected fractions were lyophilised to afford the pure products. The fractions containing impurity **2** were collected at retention time 9.4 min and those containing impurity **3** were collected at retention time 8.7 min. The respective fractions were combined and the solvent removed using a lyophiliser (Labconco® FreeZone® Plus™ 12 L) to obtain impurities **2** and **3**.

NMR, LC-MS, MS, IR and melting points apparatus

NMR spectra were obtained on a Bruker Avance III spectrometer (^{1}H NMR at 600 Hz, ^{13}C NMR at 150 Hz). Chemical shifts (δ) are given in ppm and coupling constants (*J*) are given in Hertz (Hz). The solvents used were $CDCl_3$, CD_3OD or DMSO-d_6. The mass spectra and high-resolution mass spectra were recorded on an Aglient 6210B series single quadrupole LC-MS and a Q-Tof micro YA019 instrument. Melting points were measured on a WRS-1B apparatus. FTIR spectra were recorded in the solid state using attenuated total reflectance method on IRT racer-100 (Shimadzu Corporation, Kyoto, Japan).

*Synthesis of (2*R*,6*R*,7*S*)-3-((carbamoyloxy)methyl)-7-methoxy-8-oxo-7-(2-(thiophen-2-yl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid* (**2**)*; general procedure*

Triethylamine (4.7 g, 46.5 mmol) was added to a solution of cefoxitin **1** (10.0 g, 23.4 mmol) in methanol (100 mL) and stirred for 24 h at room temperature. HPLC showed about 13% of **1** had been converted to the double-bond isomer **2**. The solvent of the reaction mixture was removed under reduced pressure at 25 °C. The residue was dissolved in ethyl acetate (150 mL) and washed with 5% aqueous hydrochloric acid (100 mL) to remove triethylamine. The ethyl acetate layer was washed with water (80 mL) and concentrated under reduced pressure to remove organic solvent. The crude oil was further purified by semi-preparative liquid chromatography to obtain **2** a pale yellow solid; purity 98.8%; yield 800 mg (8%); m.p. 125–130 °C; ^{1}H NMR (600 MHz, CD_3OD): δ 7.29 (dd, ^{1}H, J_1 = 1.2 Hz, J_2 = 5.4 Hz), 7.01 (m, 1H), 6.97 (dd, ^{1}H, J_1 = 3.6 Hz, J_2 = 5.4 Hz), 6.25 (s, 1H), 5.50 (s, 1H), 4.80 (s, 1H), 4.77 (d, *J* = 12.6 Hz, 1H), 4.62 (d, *J* = 12.6 Hz, 1H), 3.87 (q, *J* = 15.6 Hz, 2H), 3.50 (s, 3H); ^{13}C NMR (150 MHz, CD_3OD): δ 174.7, 174.0, 162.6, 160.0, 137.5, 128.5, 128.3, 126.5, 124.3, 120.5, 97.5, 67.9, 61.5, 54.9, 54.3, 38.0. HRMS *m/z* calcd for $C_{16}H_{17}N_3O_7S_2$ $[M + Na]^+$: 450.0400; found: 450.0404.

*Synthesis of (6*R*,7*S*)-3-((carbamoyloxy)methyl)-7-methoxy-7-((*R*)-2-methoxy -2-(thiophen-2-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid* (**3**)*; general procedure*

Methylene chloride (150 mL), methanol (15 mL) and cephalothin **5** (30 g, 75.7 mmol) were added to a 500 mL three-necked flask. The reaction mixture was cooled to −70 ± 5 °C in a liquid nitrogen bath, and then sodium methoxide solution (5.4 mol L^{-1}; 84.1 mL, 454.1 mmol) was added into the solution at −70 ± 5 °C. Next, *t*-butyl hypochlorite (20.5 g, 188.8 mmol) was slowly added dropwise to control the temperature at −70 ± 5 °C. After the reaction was completed, the reaction mixture was treated with sodium metabisulfite (28.7 g, 107.8 mmol) and stirred for 10 min at −70 °C. The pH value of the solution was adjusted to 6–7 at −60 °C with acetic acid and then further adjusted to pH 1–2 with 10% hydrochloric acid at 0–5 °C. The aqueous layer was separated and was extracted again with dichloromethane (100 mL). The combined organic phase was washed once with water (100 mL) and the organic phase was treated with saturated sodium bicarbonate solution to adjust the aqueous phase to pH 7–8. The organic phase was discarded. The obtained aqueous phase was adjusted carefully to pH 1–2 with 10% hydrochloric acid and extracted with ethyl acetate (200 mL). The organic phase was concentrated to generate **7** a light brown oil, which was not further purified for the next step; yield 24.6 g; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.63 (d, *J* = 21.6 Hz, 1H), 7.27–7.25 (m, 1H), 7.11 (dd, J_1 = 3 Hz, J_2 = 17.4 Hz, 1H), 6.95–6.92 (m, 1H), 5.06–5.01 (m, 3H), 4.95 (d, *J* = 7.8 Hz, 1H), 4.88 (d, *J* = 14.4 Hz, 1H), 3.52–3.35 (m, 7H), 3.29–3.19 (m, 1H), 2.02 (d, 3H, *J* = 4.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.0, 169.8, 162.1, 159.7, 130.1, 129.2, 126.8, 126.3, 126.0, 125.9, 125.5, 125.4, 124.6, 124.5, 93.9, 78.7, 78.4, 63.4, 63.3, 61.8, 56.7, 56.4, 52.9, 52.7, 25.8, 19.7; MS (ESI) *m/z*: 474.14 $[M + H_2O]$.

Sodium hydroxide solution (6.3 g NaOH dissolved in 40 mL water) was added at −30 °C to a mixture of water (50 mL), methanol (100 mL) and crude **7** (24.0 g) cooled to −30 °C, and the solution was stirred for 3 h. After completion of the hydrolysis reaction, the pH was adjusted to 6–7 using acetic acid at −30 °C. The reaction temperature was raised to 30 °C. The solvent was distilled off under reduced pressure, followed by a solvent switch to tetrahydrofuran. Anhydrous magnesium sulfate was used to dry the tetrahydrofuran solution of compound **10**. The solvent was concentrated under reduced pressure to obtain a solid (30.0 g), which was directly used in the next step without purification.

Crude compound **10** (30.0 g) in tetrahydrofuran was cooled to −40 °C under liquid nitrogen, and a solution of chlorosulfonyl isocyanate (22.3 g, 157.6 mmol) was slowly added at −40 °C. The reaction was monitored by HPLC. When the reaction was completed, the reaction mixture was added into ice water and stirred for 3 h. Tetrahydrofuran was removed under reduced pressure. The product was extracted with ethyl acetate and the aqueous phase discarded. The organic layer was washed with saturated sodium chloride solution. The organic phase was treated with sodium bicarbonate solution to adjust the aqueous phase to pH 7–8. The obtained aqueous phase was adjusted carefully to pH 1–2 with 10% hydrochloric acid and extracted into ethyl acetate. Ethyl acetate was distilled off under reduced pressure and crude **3** [yield 11.1 g (32%)] was obtained. The crude **3** (1.0 g) was further purified by semi-preparative liquid chromatography to obtain **3** as a pale yellow solid; purity 96%; yield 400 mg; m.p. 53–55 °C; ^{1}H NMR (600 MHz, CD_3OD): δ 7.46–7.44 (m, 1H), 7.24–7.22 (m, 1H), 7.04–7.02 (m, 1H), 5.14 (s, 0.5H), 5.10 (s, 0.5H), 5.08 (d, *J* = 1.2 Hz, 1H), 5.05 (dd, J_1 = 2.4 Hz, J_2 = 13.8 Hz, 1H), 4.84 (dd, J_1 = 4.2 Hz, J_2 = 13.2 Hz, 1H), 3.59–3.55 (m, 1H), 3.52 (s, 1.5H), 3.47 (d, *J* = 4.2 Hz, 3H), 3.43 (s, 1.5H), 3.37–3.30 (m, 1H); ^{13}C NMR (150 MHz, CD_3OD): δ 172.0, 163.6, 160.5, 158.0, 139.0, 128.9, 128.5, 127.3, 127.1, 126.6, 126.4, 126.3, 126.2, 123.5, 95.7, 95.6, 79.3, 78.7, 64.2, 62.7, 56.5, 56.4, 52.6, 52.5, 25.7. HRMS *m/z* calcd for $C_{17}H_{19}N_3NaO_8S_2$ $[M + Na]^+$: 480.0506; found: 480.0511.

Synthesis of N-*((5a*R*,6*S*)-6-methoxy-1,7-dioxo-1,4,5a,6-tetrahydro-3H,7H-azeto[2,1-b]furo[3,4-d][1,3]thiazin-6-yl)-2-(thiophen-2-yl) acetamide* (**4**)*; general procedure*

Methylene chloride (100 mL), methanol (10 mL) and cephalothin **5** (10.0 g, 25.5 mmol) were added to a 250 mL three-necked flask. The reaction mixture was cooled to −70 ± 5 °C in a liquid nitrogen bath, and then sodium methoxide solution (5.4 mol L^{-1}; 23.6 mL, 127.5 mmol) was added into the mixture at −80 °C. Next, *t*-butyl hypochlorite (3.3 g, 30.6 mmol) was slowly added dropwise to control the temperature at −80 °C. After the reaction was completed, the reaction mixture was treated with sodium metabisulfite (7.3 g, 38.4 mmol) and stirred for

10 min at −70 °C. The pH value of the solution was adjusted to 6–7 at −60 °C with acetic acid and then further adjusted to pH 1–2 with 10% hydrochloric acid at 0–5 °C. The aqueous layer was separated and was extracted again with dichloromethane (50 mL). The combined organic phase was washed once with water (100 mL) and the organic phase was treated with saturated sodium bicarbonate solution to adjust the aqueous phase to pH 7–8. The organic phase was discarded. The obtained aqueous phase was adjusted carefully to pH 1–2 with 10% hydrochloric acid and extracted with ethyl acetate (100 mL). The organic phase was washed with water (50 mL) and dried over anhydrous sodium sulfate. A solution of cyclohexylamine (3 g, 30.2 mmol) in acetone (5 mL) was added to the ethyl acetate extract at 25 °C. Intermediate **11** was precipitated from the reaction solution. The isolated solid, a cyclohexylamine salt, was collected by filtration and dried to yield **11** as a pale yellow solid; yield 10.3 g (78.1%); m.p. 139–142 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.39 (s, 1H), 8.22 (br, s, 3H), 7.37 (dd, J_1 = 1.2 Hz, J_2 = 4.8 Hz, 1H), 6.96–6.94 (m, 2H), 4.97 (s, 1H), 4.86 (d, J = 12.0 Hz, 1H), 4.68 (d, J = 12.0 Hz, 1H), 3.80 (q, J = 9.0 Hz, 2H), 3.44 (d, J = 18.0 Hz, 1H), 3.34 (s, 1H), 3.07 (d, J = 18.0 Hz, 1H), 2.94–2.90 (m, 1H), 1.99 (s, 3H), 1.91–1.89 (m, 3H), 1.71–1.68 (m, 2H), 1.57 (d, J = 13.6 Hz, 1H), 1.30–1.19 (m, 4H), 1.11–1.06 (m, 1H); ^{13}C NMR (150 MHz, DMSO-d_6): 170.9, 164.1, 159.6, 137.3, 135.4, 127.1, 126.9, 125.2, 111.9, 94.9, 64.6, 62.7, 60.2, 52.7, 49.6, 36.4, 30.9, 25.6, 25.1, 24.3, 21.1, 14.6. HRMS m/z calcd for $C_{17}H_{18}N_2NaO_7S_2$ [M + Na]$^+$: 449.0448; found: 450.0451.

Sodium hydroxide solution (1.1 g NaOH dissolved in 20 mL water) was added at −30 °C to a mixture of water (20 mL), methanol (20 mL) and crude **11** (5.0 g) cooled to −30 °C, and the solution was stirred for 2 h. After completion of the hydrolysis reaction, the pH was adjusted to 6–7 using acetic acid at −30 °C. The reaction temperature was raised to 0–5 °C. The reaction solution was further adjusted to pH 1–2 with 10% hydrochloric acid and stirred at 5–10 °C for 3 h. The solvent methanol was removed under reduced pressure and crude **4** extracted with ethyl acetate (80 mL). Ethyl acetate was concentrated and the residue was purified by silica gel column chromatography (ethyl acetate:*n*-hexane = 1:1) to yield **4** as a pale yellow solid; yield 2.6 g (73.6%); m.p. 161–162 °C; IR (ν cm^{-1}): 1789 (C=O), 1751 (C=O), 1666.5 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.54 (s, 1H), 7.38 (dd, J_1 = 1.2 Hz, J_2 = 5.4 Hz, 1H), 6.97–6.94 (m, 2H), 5.17 (s, 1H), 5.02 (s, 2H), 3.82 (q, J = 15.6 Hz, 2H), 3.76 (d, J = 18.6 Hz, 1H), 3.65 (d, J = 18.6 Hz, 1H), 3.41 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6): 170.1, 166.5, 159.6, 142.9, 137.0, 127.1, 127.0, 125.6, 123.0, 96.3, 71.9, 63.1, 53.0, 36.3, 23.3. HRMS m/z calcd for $C_{15}H_{14}N_2NaO_5S_2$ [M + Na]$^+$ 389.0236; found: 389.0240.

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