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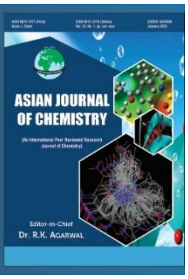

Synthesis and Characterisation of Critical Impurities in Ampicillin Trihydrate: N-Formyl Ampicilloic Acid and D-Phenylglycylampicillin

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The current research work elucidates the identification, synthesis and characterisation of two critical impurities of ampicillin trihydrate, designated as *N*-formyl ampicilloic acid and D-phenylglycylampicillin (European Pharmacopoeia impurity I). The formation of *N*-formyl ampicilloic acid results from the cross-contamination of ampicillin trihydrate with residual formic acid, whereas D-phenylglycylampicillin arises through the amidation of amino group in the side chain of ampicillin trihydrate with D-phenylglycine methylester. These impurities substantially affect the overall quality of the ampicillin trihydrate API; therefore, their identification and control are critical. This work holds significant value for the generic pharmaceutical industry, as an efficient synthetic route for these impurities has been established using readily accessible starting materials.

Keywords: Ampicillin trihydrate, *N*-Formyl ampicilloic acid, D-Phenylglycylampicillin.

INTRODUCTION

Ampicillin is a semisynthetic penicillin-class antibiotic, characterised by its broad-spectrum antimicrobial activity. It is one of the most extensively utilised β-lactam antibiotics, commonly prescribed for managing infections caused by various pathogens. Ampicillin is frequently co-administered with clavulanic acid, a β-lactamase inhibitor to counteract microbial resistance mechanisms involving β-lactamase enzyme [1,2]. It is used to treat a variety of bacterial infections, indications include urinary tract infections, respiratory tract infections, meningitis, salmonella infections and endocarditis [3]. This product can also be used to prevent group B streptococcal infections in neonates and can be administered orally, intravenously and intramuscularly [3]. Aminopenicillins also include amoxicillin, which are easier to penetrate Gram-negative bacteria than penicillins or penicillinase-resistant penicillins, but are easily decomposed by bacterial β-lactamases, so they are often used in combination with sulbactam (β-lactamase inhibitor) to enhance the therapeutic effect on bacteria that can produce β-lactamases [4].

Ampicillin was first developed by Bristol Myers Co in 1961 and is listed in World Health Organisation's Standard List of Essential Medicines and is one of the essential drugs for the basic public health system. Ampicillin is officially documented in the monographs of both the US and European Pharmacopoeias [5,6]. Numerous synthetic pathways (enzymatic and chemical synthetic process) for the production of ampicillin trihydrate (**1**) have been described in the literature [7-9]. Conveniently, ampicillin trihydrate was synthesised (chemical synthetic route) by using commercially available precursors, namely D-phenylglycine (D-PHG) and 6-aminopenicillanic acid (6-APA) [10,11]. In brief, D-phenylglycine (**4**) was reacted with ethyl acetoacetate using K_2CO_3 as a base in methanol forming dane salt (**A**) as an *in situ* intermediate. The dane salt (**A**) was subsequently reacted with ethyl chloroformate in the presence of pyridine and MDC to generate the mixed anhydride (**B**) as another *in situ* intermediate. Meanwhile, 6-APA.TEA (**C**) was prepared by reacting 6-APA (**5**) with TEA in MDC. This intermediate (6-APA.TEA) (**C**) was then reacted with mixed anhydride (**B**) to yield *N*-protected-D-ampicillin·TEA (**D**). Finally, intermediate **D** underwent acid-base treatment to produce ampicillin trihydrate (**1**) (**Scheme-I**).

Thus, during the synthesis of ampicillin trihydrate (**1**), contaminants **2** and **3** were identified. The formation of

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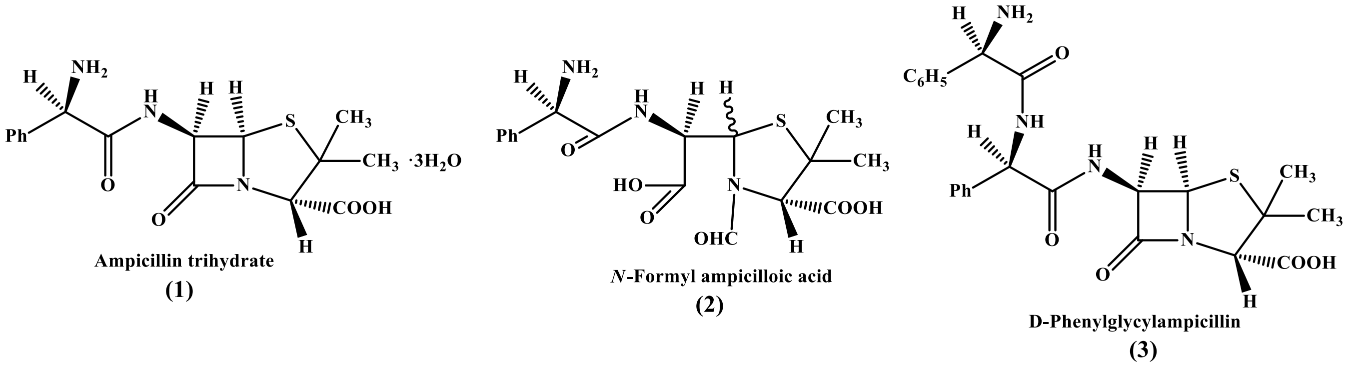
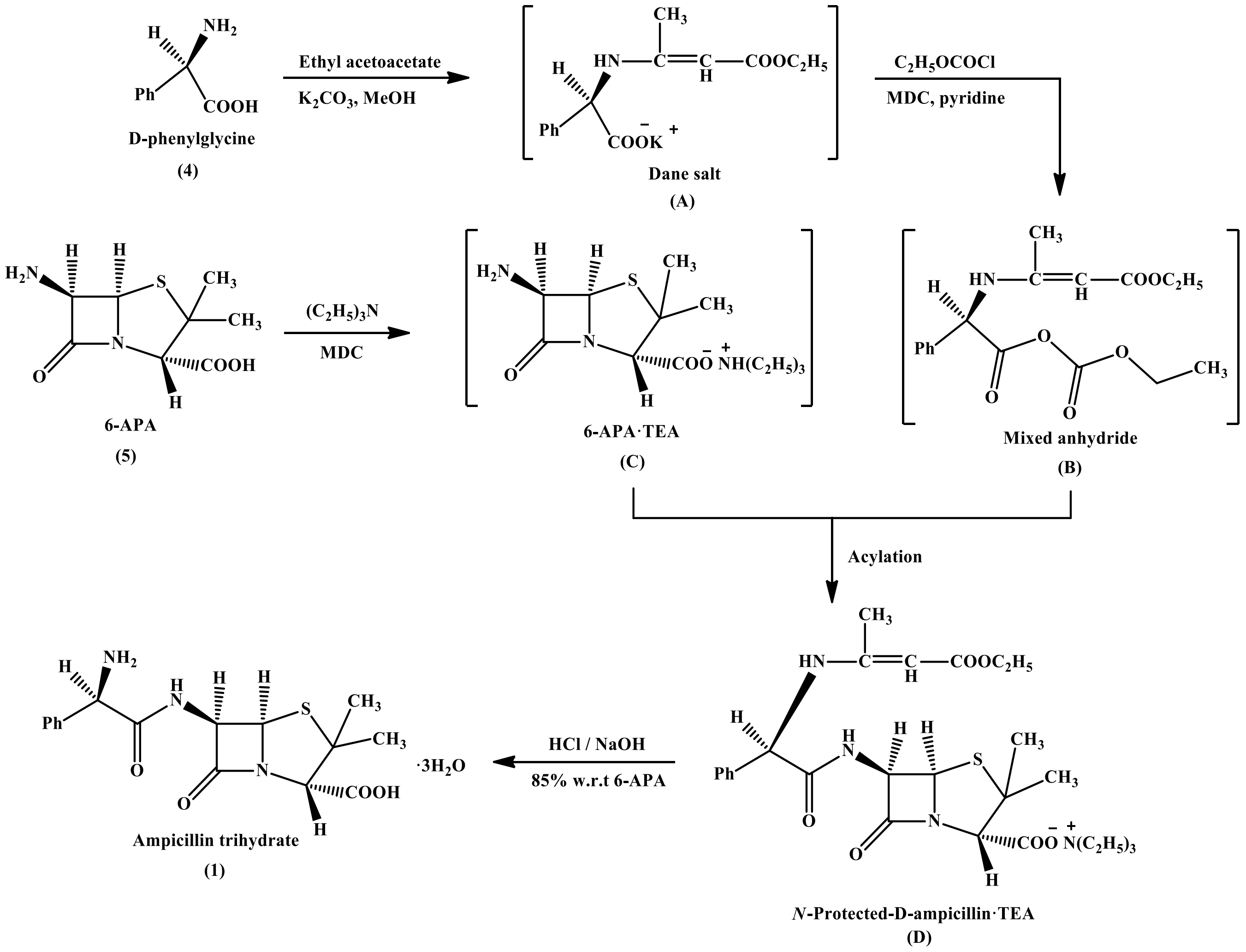


Ampicillin trihydrate
(1)

***N*-Formyl ampicilloic acid**
(2)

D-Phenylglycylampicillin
(3)

Fig. 1. Chemical structures of ampicillin trihydrate (**1**) and its related impurities (**2** and **3**)



Scheme-I: Synthesis of ampicillin trihydrate (**1**)

impurity *N*-formyl ampicilloic acid (**2**) occurs *via* during the hydrolytic cleavage of the β-lactam ring in the penicillin nucleus of ampicillin trihydrate (**1**) followed by a concurrent reaction with residual formic acid. Impurity **3** originates from the amidation reaction between the amino group in the side chain of ampicillin trihydrate (**1**) and D-phenylglycine methylester leading to the formation of contaminant **3** (D-phenylglycylampicillin).

A primary challenge for organic chemists lies in developing high-quality, reproducible and safe synthetic routes within the constraints of the developmental process. Furthermore, identifying impurities or related substances that form in trace amounts during the synthesis or manufacturing of a drug substance remains a significant hurdle. The presence of these impurities in an active pharmaceutical ingredient (API) can greatly influence the drug's quality and safety. As per the

United States Pharmacopeia (USP 40), the threshold limit of a known or unknown limit of contaminants in drug substance (API) should be $\leq$ 0.10%. Thus, these two contaminants **2** and **3** of ampicillin trihydrate were synthesized and characterised these using spectroscopic techniques by NMR (^{1}H and ^{13}C) and HRMS analysis.

EXPERIMENTAL

The proton NMR (^{1}H NMR) and ^{13}C NMR spectra were recorded using a Bruker Advance 300 MHz and a Varian 500 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. Chemical shift values were reported on the δ-scale relative to TMS and referenced against deuterated solvents such as D_2O, DMSO-d_6 and $CDCl_3$ as internal standards. High-resolution mass spectrometry (HRMS) was performed using the electrospray ionisation (ESI) method on a Xevo G2 QTOF mass spectrometer. All raw materials were obtained from commercial suppliers and used as received without any further purification.

Synthesis of (2*RS*,4*S*)-2-[[(1*R*)-1-carboxy-1-[(2*R*)-2-amino-2-phenylacetyl]-amino]methyl]-5,5-dimethyl-3-formyl-1,3-thiazolidine-4-carboxylic acid (*N*-formyl ampicilloic acid) (2): Ampicillin trihydrate (**1**) (10 g, 24.78 mmol) was charged into a four-necked round-bottom flask at room temperature. Formic acid (2.28 g, 49.57 mmol) was added at ambient temperature. The reaction mixture was then heated to 40-45 ºC and stirred for 6 h, during which the initially solid reaction mass was transformed into a suspension. Upon completion of the reaction, the mixture was cooled to 20-30 ºC and added methanol (50 mL) slowly in 30 min at 20-30 ºC. The resulting suspension was stirred for 1 h at 20-30 ºC, filtered the solid and the residue was washed with methanol (10 mL) (**Scheme-II**). The product was dried under vacuum at a temperature below 50 ºC yielding compound **2** (7 g, 71.43%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm); 1.13, 1.26, 1.28 & 1.38 (4s, 6H), 3.90 & 4.08 (2s, 1H), 4.48 & 4.59 (2d, 1H, *J* = 6 Hz), 4.70 & 4.84 (2s, 1H), 5.51 & 5.53 (2d, 1H, *J* = 4.8 Hz & 6.6 Hz), 7.25-7.48 (m, 5H), 8.12, 8.14, 8.34 & 8.38 (2s, 1H), 8.18 & 8.59 (2brs, 1H); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 25.97, 27.67, 27.83, 31.77, 49.12, 53.59, 56.17, 56.29, 57.01, 57.19, 59.71, 64.86, 71.55, 72.94, 127.48, 127.72, 128.12, 128.22, 138.63, 161.04, 163.00, 168.27, 170.01, 170.04, 170.37, 170.41, 170.71; HRMS (ESI-QTOF) for $C_{17}H_{21}N_3O_6S$ $[M+H]^+$: *m/z* calcd.: 396.1224; found: 396.1220; Specific optical rotation: +12.698º, $[\alpha]^{20}D$ (c = 0.25, in water).

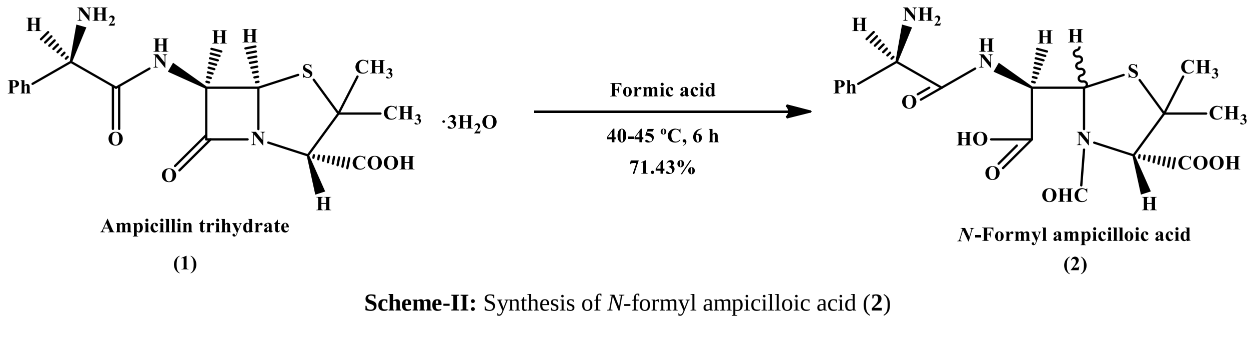
Synthesis of (2*S*,5*R*,6*R*)-6-((*R*)-2-amino-2-phenylacetamido)-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin) (3)

Synthesis of dane salt (A): Potassium carbonate (14.63 g, 105.84 mmol) was introduced into a suspension of ethyl acetoacetate (15.15 g, 116.42 mmol) and D-phenylglycine (**6**) (16 g, 105.84 mmol) in methanol (160 mL) at 20-30 ºC. The reaction mixture was then heated to reflux and stirred for 1 h, while simultaneously collecting 80 mL of methanol distillate at atmospheric pressure between 65-70 ºC. Subsequently, the reaction mixture was cooled to 20-30 ºC and stirred for 1 h, followed by further cooling to 0-5 ºC and stirring for an additional 1 h. The resulting precipitate was isolated by filtration and dried under vacuum to afford dane salt (**A**) as an off-white solid (16.43 g, 52% yield).

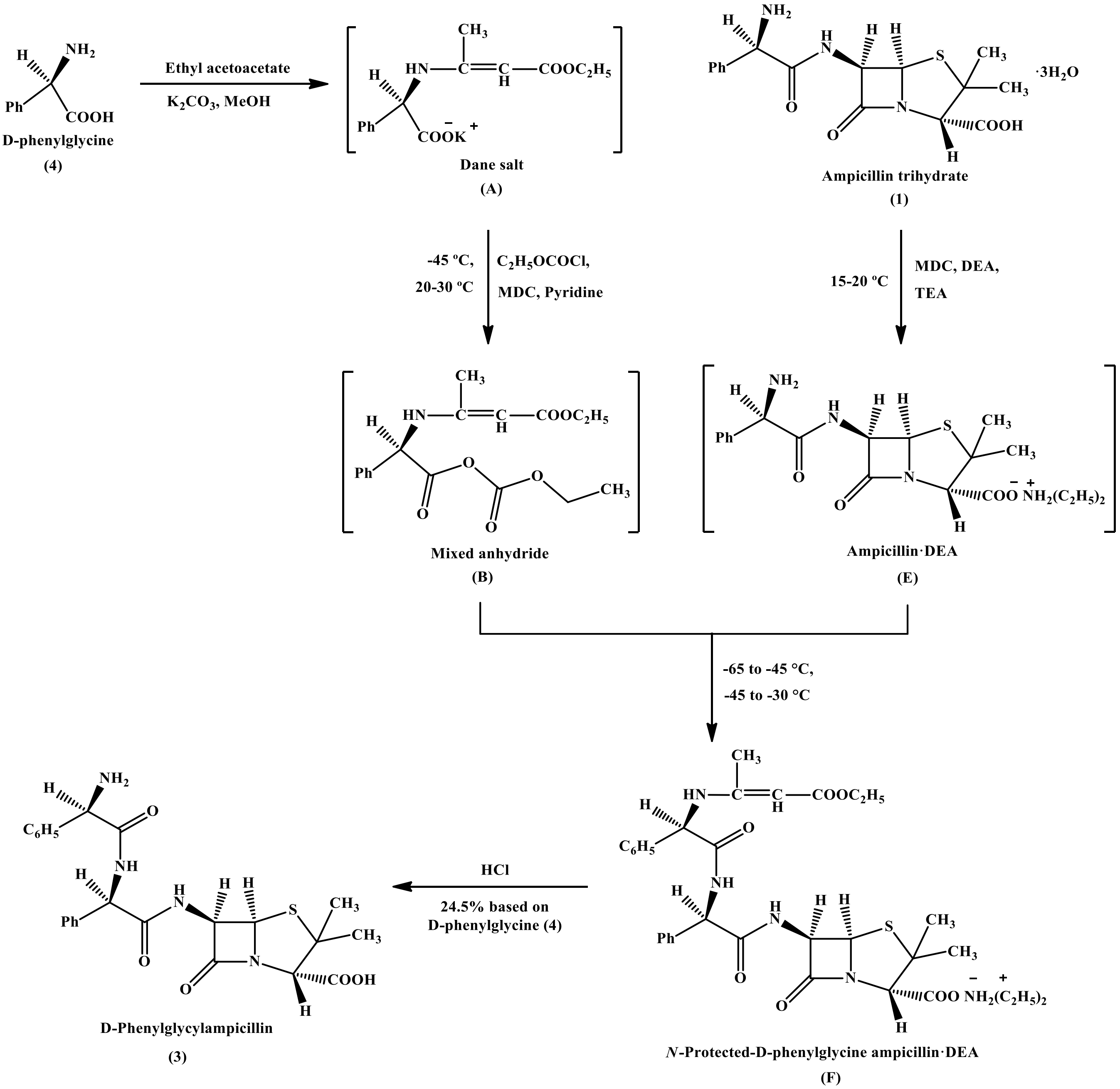
Synthesis of mixed anhydride (B): Dane salt (**A**) (16.4 g, 54.48 mmol) was dissolved in MDC (50 mL) at 20-30 ºC, then cooled to -45 ºC -40 ºC. Pyridine (0.16 g, 1% w/w) and ethyl chloroformate (5.91 g, 54.48 mmol) were added dropwise under nitrogen atmosphere. The resulting mixture was stirred for 60 min at -20 to -25 ºC to complete the formation of mixed anhydride **B**. The resulting mixed anhydride was utilised directly without any further purification.

Synthesis of ampicillin·DEA (E): Ampicillin trihydrate (**1**) (20 g, 49.62 mmol) was suspended in MDC (80 mL) at 10-15 ºC. Diethylamine (3.99 g, 54.59 mmol) was added at 10-15 ºC followed by the addition of catalytical amount of TEA (0.3 g, 1.5% w/w) at 10-20 ºC. The reaction mass was stirred for 1 h to obtain a clear solution. The resulting salt was used directly without any further purification.

Synthesis of D-phenylglycylampicillin (3): Ampicillin· DEA salt (**E**) was added dropwise to the freshly synthesized mixed anhydride (**B**) at -60 ºC to -50 ºC under stirring. The reaction mixture was stirred for 3 h to ensure the formation of *N*-protected-D-phenylglycine ampicillin·DEA (**F**) salt as an *in situ* intermediate. After completion of the reaction, precooled water (120 mL, 10-15 ºC) was added and the temperature was promptly raised to 0-2 ºC. HCl (12 mL, 35% w/w) was then added to adjust the pH to ~0.8 at 5-10 ºC and the resulting suspension was stirred for 1 h at 5-10 ºC (**Scheme-III**). The precipitated solid was collected by filtration, washed with water (40 mL) and dried under vacuum to afford D-phenylglycylampicillin (**3**) as a white solid (12.6 g, 24.5% based on D-phenylglycine (**4**). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 1.38 (s, 3H), 1.50 (s, 3H), 4.17 (s, 1H), 5.10 (s, 1H), 5.34 (d, 1H, *J* = 3.9 Hz), 5.45 (dd, 1H, *J* = 3.9 Hz & 7.8



Scheme-II: Synthesis of *N*-formyl ampicilloic acid (**2**)



Scheme-III: Synthesis of D-phenylglycylampicillin (**3**)

Hz), 5.76 (d, 1H, *J* = 8.1 Hz), 7.30-7.33 (m, 3H), 7.42-7.50 (m, 5H), 7.53-7.55 (m, 2H), 8.1-9.0 (brs, 2H), 9.14-9.17 (d, 1H, *J* = 7.8 Hz), 9.25-9.28 (d, 1H, *J* = 7.5 Hz); ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm): 26.65, 30.59, 54.98, 55.69, 58.21, 63.85, 67.19, 70.33, 127.26, 127.90, 128.31, 128.80, 129.13, 134.17, 137.62, 167.01, 168.88, 169.25, 173.10 ppm; HRMS (ESI-QTOF) for $C_{24}H_{26}N_4O_5S$ $[M+H]^+$: *m/z* calcd.: 483.1697; found: 483.1711; Specific optical rotation: +61.870°, $[\alpha]^{20}_D$ (c = 0.25, in methanol).

RESULTS AND DISCUSSION

During the scale-up studies of ampicillin trihydrate (**1**) in the kilo lab, the impurity of *N*-formyl ampicilloic acid (**2**) was identified at concentrations ranging from 0.5 to 3% as detected by HPLC and LC-MS. Significant challenges were encountered in determining the origin of this impurity. Subsequently, impurity **2** was isolated using preparative HPLC and its structure was characterised and confirmed as *N*-formyl ampicilloic acid (**2**) through various spectroscopic techniques including ^{1}H NMR, ^{13}C NMR and mass spectrometry. Further, investigation revealed that the formation of impurity **2** resulted from the cross-contamination of residual formic acid present in the reactor lines. Then, several chemical methods were explored to synthesize impurity **2** directly, ultimately achieving its preparation without the use of preparative HPLC, column purification and recrystallisation. The synthesis of *N*-formyl ampicilloic acid (**2**) was carried out independently by reacting ampicillin trihydrate (**1**) with formic acid at 40-45 ºC for 6 h (**Scheme-II**). This method features a straightforward synthetic pathway and mild reaction conditions (neat reaction) yielding impurity **2** with high purity and good efficiency with specific optical rotation of +12.698º. To the best of our knowledge, there are no prior reports on the synthesis of *N*-formyl ampicilloic acid (**2**).

The synthetic pathway for D-phenylglycylampicillin (**3**) is illustrated in **Scheme-III**. In this process, D-phenylglycine (**4**) was reacted with ethyl acetoacetate in the presence of mild inorganic base K_2CO_3 in methanol leading to the formation of dane salt (**A**). Concurrently, ampicillin·DEA salt (**E**) was prepared by reacting ampicillin trihydrate (**1**) with diethylamine (DEA) in MDC using a catalytical amount of triethylamine (TEA) to obtain a clear solution. Then, acylation of ampicillin·DEA salt (**E**) with the mixed anhydride (**B**) resulted in the formation of *N*-protected-D-phenylglycine ampicillin·DEA salt (**F**) as an *in situ* intermediate. Finally, deprotection of the *N*-protected-D-phenylglycine ampicillin·DEA salt (**F**) using HCl yielded D-phenylglycylampicillin (**3**) with specific optical rotation of +61.870º.

D-Phenylglycylampicillin (**3**) synthesis was previously reported by Zhongbo *et al.* [3]. In their synthetic route, D-phenylglycine was reacted with methyl acetoacetate in presence of strong base KOH in ethanol to form dane salt (**A**). In contrast, D-phenylglycylampicillin (**3**) was synthesised using a different approach, employing K_2CO_3 as a base, methanol as a solvent and an alternative synthetic methodology. While acetone and DMF were used in the reference synthesis to obtain the mixed anhydride (**B**), we used MDC as a solvent and pyridine as a base for this step. Furthermore, the reference method utilised ampicillin·ammonia solution to synthesise an *in situ* intermediate. In contrast, our approach employed ampicillin·DEA salt (**E**) to synthesize the *in situ* intermediate (**F**) [3,12].

Conclusion

A synthetic methodology for synthesising two significant potential impurities of ampicillin trihydrate (**1**) using commercially available key starting materials. The impurities were characterised by ^{1}H NMR, ^{13}C NMR and HRMS analysis. This approach is adaptable for synthesizing other penicillin derivatives with diverse substituents. These impurities are intended to serve as reference standards in the drug regulatory approval process and support in finalizing the specifications of ampicillin trihydrate (**1**).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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