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Identification of a diastereomer of penicilloic acid during acid-mediated degradation of 6-aminopenicillanic acid

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ABSTRACT

6-Aminopenicillanic acid (6-APA), the core scaffold of penicillin antibiotics, is a key intermediate in the industrial synthesis of β-lactam drugs but it is prone to degradation under acidic conditions due to the strain of the β-lactam ring. Here, the acid-mediated degradation of 6-APA was investigated by real-time ^{1}H NMR, complemented by HPLC and High-resolution mass spectrometry. An unexpected degradation product was detected that did not fully correspond to the classical β-lactam hydrolysis pathways. Structural analysis revealed that the species corresponds to a diastereomer of penicilloic acid, consistent with epimerization at the C6 position following β-lactam hydrolysis. This integrated analytical approach provides the first direct NMR evidence of a diastereomeric transformation pathway under acidic conditions, offering new insights into the stability of 6-APA that are relevant to pharmaceutical analysis and process control.

Introduction

6-Aminopenicillanic acid (6-APA) is the common structural core of all semisynthetic penicillins and is a key intermediate in the industrial production of β-lactam antibiotics (Fig. 1).[1–3] The biological activity of these compounds depends critically on the integrity of the β-lactam ring, a highly strained four-membered amide which is prone to hydrolytic degradation.[4] Consequently, the stability of 6-APA during processing and storage is of considerable practical importance. Both acidic and basic conditions promote β-lactam ring opening, although alkaline environments generally lead to faster hydrolysis, with consequent loss of biological activity and the formation of structurally modified derivatives. Despite extensive studies on β-lactam degradation, transformation pathways of 6-APA under acidic conditions have not been fully characterised, particularly with respect to transient intermediates and stereochemical outcomes.[5–8]

Previous studies on 6-APA have mainly focused on establishing broad impurity profiles. However, direct, time-resolved NMR evidence of stereochemical distinct products under acidic conditions remains limited.[7] In particular, although related species have been proposed, direct NMR characterization of such diastereomers under acid conditions has not been established. Herein, we report a time-resolved investigation of the acid-mediated degradation of 6-APA by ^{1}H NMR spectroscopy complemented by HPLC and high-Resolution mass spectrometry (HRMS). During these studies, we observed the formation of a degradation product that had not been reported previously, and which does not fully correspond to classical acidic β-lactam hydrolysis pathways.[3, 4] Structural analysis revealed that this species corresponds to a diastereomer of penicilloic acid, consistent with epimerization at C6. To the best of our knowledge, this compound has not previously been characterized by NMR nor reported under acidic degradation conditions.

Materials and methods

All reagents were used as received without further purification. The reported DCl concentrations refer to the nominal concentrations obtained by dilution of the stock solution.

NMR Experiments

^{1}H NMR spectra were recorded on a 600 MHz spectrometer at 25 °C. Each spectrum was acquired using 32 scans. Stock solutions were prepared in 1 mL glass vials. For each experiment, 109 mg of 6-aminopenicillanic acid (6-APA) were dissolved in a mixture of D_2O and deuterium chloride solution (DCl; 35 wt% in D_2O) to obtain the

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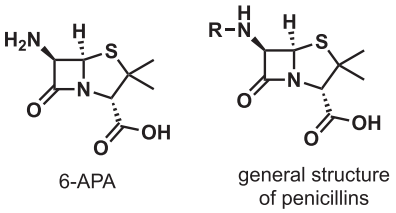

Fig. 1. Chemical structure of 6-APA and general structure of Penicillins.

desired acid concentration (0.75−2.5 M). Maleic acid was added as an internal standard to allow normalization of NMR signal integrals during the kinetic analysis. After complete dissolution, 0.6 mL of the reaction mixture was transferred into an NMR tube, and spectra were recorded at different time intervals in order to monitor the degradation process in real time.

All spectra were acquired under identical experimental conditions to ensure reliable comparison of signal integrals. All spectra were processed using identical phase and baseline correction parameters. The integrals of diagnostic signals of 6-APA and the degradation products were normalized with respect to the maleic acid signal and used for kinetic analysis. Chemical shifts were referenced to the signal of maleic acid.

Integration of the diagnostic resonances was performed using identical integration regions for all spectra to ensure consistency throughout the time-resolved experiments.

HPLC Degradation studies

Stability Stock Solution preparation: approximately 1080 mg of 6-APA batch 1003585262 (Sigma Aldrich. Assay 99.6%) are weighted in 20 mL volumetric flasks and are diluted with 10 mL of water. Different amounts of HBr 48% are added to solubilize the analyte and then solution is brought to volume with water. HBr was selected for the HPLC degradation studies due to its compatibility with the analytical method and its ability to ensure efficient solubilization of 6-APA under the selected conditions.

Sample Solution preparation: 1 mL of Stability Stock Solution is diluted in 100 mL volumetric flasks with diluent H_2O/ACN/HBr 48% 75/25/0.08 (v/v/v) at different time points: 0, 15, 30, 45, 60, 90, 120, 240 min.

Standard preparation: approximately 50 mg of 6-APA batch 202210679 (Coreychem. Assay 99.8%) are weighted in 50 mL volumetric flasks and diluted with approximately 40 mL of diluent H_2O/ACN/HBr 48% 75/25/0.08 (v/v/v).

Solution is sonicated for approximately 1 min until complete solubilization and then is brought to volume with diluent.

Chromatographic analyses were performed using a UPLC system equipped with both a diode array detector (DAD) and mass spectrometry (MS) detection. Separation was performed on an Rx-Sil column (4.6 × 100 mm. particle size 1.8 μm) maintained at 25 °C, while the autosampler temperature was set at 5 °C.

The mobile phase consisted of a buffer solution (100 mM ammonium formate, pH 4), methanol, and acetonitrile (in a ratio of 10:5:85. v/v/v), and was delivered under isocratic conditions at a flow rate of 1.5 mL per minute. Detection was performed at 225 nm, with an injection volume of 5 μL. The total analysis time was 5 min.

"Degradation %" is calculated as Assay relative percentage difference between the expected value (Assay th) and the value obtained at each degradation time point (Assay deg):

$$\text{Degradation \%} = (\text{Assay}_{deg} - \text{Assay}_{th}) / \text{Assay}_{th} * 100$$

$$\text{Assay}_{th} = 99.6\%$$

The assay was determined with external standard calibration providing quantitative information on 6-APA degradation. HPLC analysis was used as an orthogonal method to monitor degradation trends and support the results obtained by time-resolved ^{1}H NMR .

Kinetic analysis

Pseudo-first-order kinetic parameters for the acid-induced degradation of 6-APA were obtained from time-resolved ^{1}H NMR experiments. The reaction was monitored in the same NMR tube under identical experimental conditions for each acid concentration. The integrals of the diagnostic ^{1}H NMR signals corresponding to 6-APA were normalised using maleic acid as an internal standard to correct for any variations in signal intensity.

The normalised concentration of 6-APA at each time point was expressed as a fraction of the initial concentration (C/C_0). The natural logarithm of these values ($\ln(C/C_0)$) was plotted as a function of time. Under these conditions, the acid concentration largely exceeds that of 6-APA and remains effectively constant during the experiment, allowing the degradation process to be treated as pseudo-first-order.

The observed pseudo-first-order rate constants (k_obs) were obtained from the slope of the linear regression of $\ln(C/C_0)$ versus time, according to the first-order rate equation:

$$\ln(C/C_0) = -k\_obs\ t$$

The corresponding half-life values ($t_{1/2}$) were calculated using:

$$t_{1/2} = \ln(2) / k\_obs$$

Linear regression analysis was used to determine the kinetic parameters and the corresponding correlation coefficients (R^2). For each acid concentration, k_obs values were obtained from the initial linear region of the $\ln(C/C_0)$ vs time plots, where deviations from linearity due to secondary processes are minimal.

Although compound **1** is formed as an intermediate and subsequently converts into compound **2** (see Figs. 2 and 3 for structures), the kinetic analysis considered only the disappearance of the starting material. Based on the time-resolved NMR data, the system is more appropriately described as an initial acid-catalyzed hydrolysis of 6-APA followed by a reversible transformation leading to an equilibrium between compounds **1** and **2**.

While absolute quantification of compounds **1** and **2** would require qNMR analysis or independently purified standards, such approaches are less suited to time-resolved experiments due to longer acquisition times. The present study therefore focuses on the relative evolution of species over time, which can be reliably monitored using normalized NMR signal integrals.

High-resolution mass spectrometry (HRMS)

HRMS spectra were obtained with a G2XS QTof mass spectrometer equipped with an electrospray ionization (ESI) source, operating in positive ion mode. Typical acquisition parameters included: capillary voltage 3 kV, cone voltage 40 V, desolvation temperature 600 °C, source temperature 120 °C, Cone Gas Flow 50 L/h, Desolvatation Gas Flow 1000 L/h. Compound identification was based on accurate mass measurements, isotopic pattern, and comparison with expected molecular formulas and fragmentation data.

Results and discussion

The acid-catalyzed hydrolysis of 6-APA was investigated over a wide range of acid concentrations (0.75−2.5 M) by monitoring the time-dependent evolution of diagnostic NMR integrals associated with the starting material and the corresponding transformation products. The resulting kinetic profiles reveal a pronounced dependence of both reaction rate and product(s) distribution on the H^+ concentration, consistent with a degradation mechanism where protonation controls the activation of the β-lactam ring.

As can be clearly seen from the overlap of the ^{1}H NMR spectra (Figs. 2c, d, 3 and 4), the stability of 6-APA strongly depends on both the acidic concentration and the incubation time. Under low acidic conditions, the diagnostic signals of 6-APA, in particular the β-lactam protons, δ 5.54 (d, J = 4.3 Hz, 1H) and 4.96 (d, J = 4.2 Hz, 1H) ppm, and

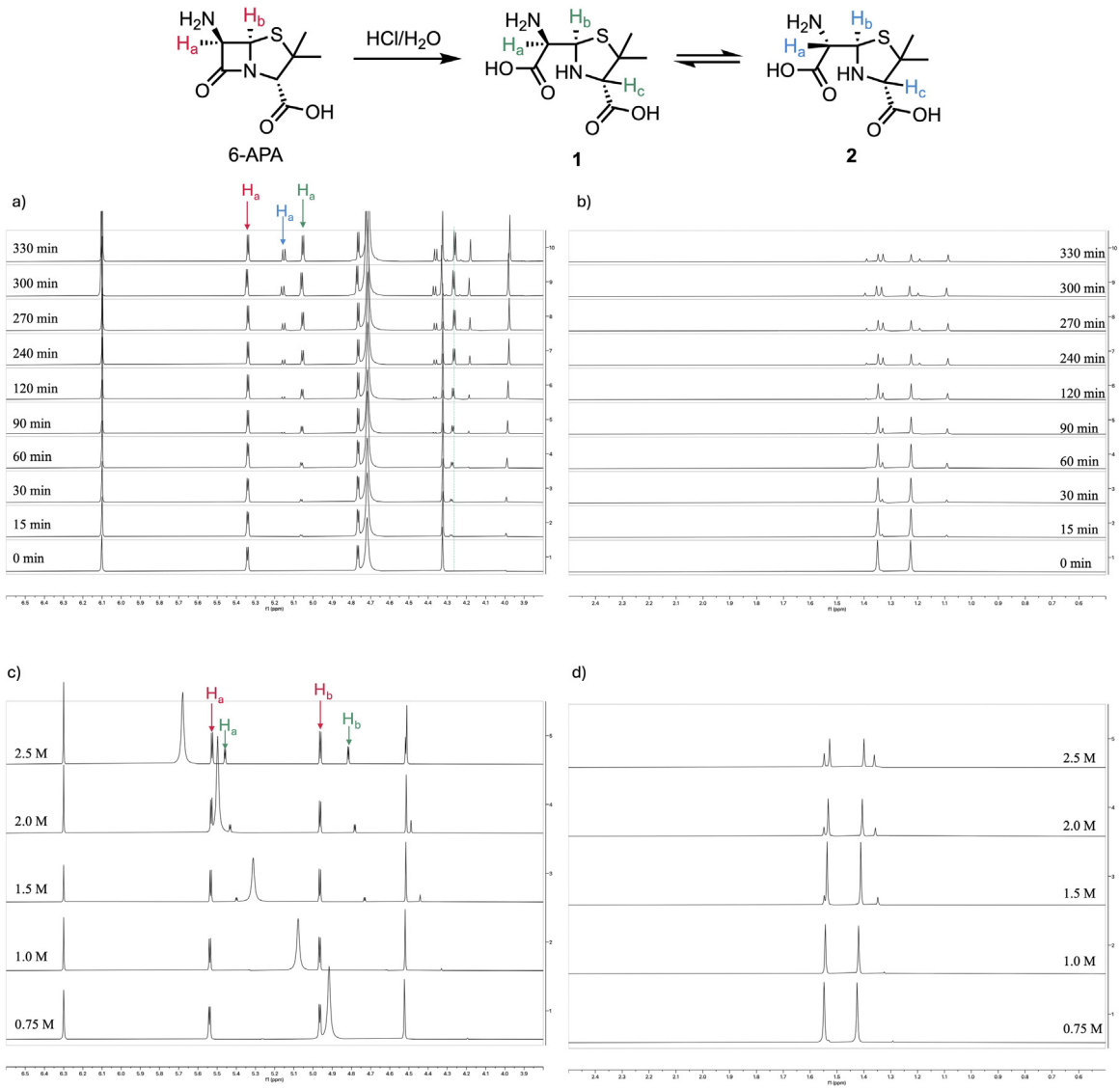

Fig. 2. Selected regions of ^{1}H NMR spectra of 6-APA under acidic conditions. a, b) Time-resolved spectra of a 0.75 M 6-APA solution in DCl/D_2O recorded at different time points; c, d) spectra recorded at t = 0 at different acid concentrations. Diagnostic resonances of 6-APA and the degradation products are indicated and color-coded (6-APA, red; compound 1, green; compound 2, blue). Only the signals relevant for assignment and kinetic analysis are shown. A complete assignment of ^{1}H NMR resonances is provided in Table SI1 and Figure SI1 (Supporting Information). Note the shift in the peak of D_2O due to the different acidic conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

the protons of the thiazolidine system, 4.52 (s, 1H), 1.55 (s, 3H), 1.43 (s, 3H) ppm, remain clearly visible at short exposure time.

However, their intensities progressively decrease as exposure time proceeds. At higher acid concentration, a more pronounced and rapid disappearance of the β-lactam proton signals is observed, indicating an accelerated degradation of the core. At the same time, we observed the appearance of new signals at δ 5.35 (d, J = 7.6 Hz) and 5.25 (d, J = 4.7 Hz) ppm. These signals could not be directly attributed to the classical β-lactam hydrolysis products described in literature.[7] Careful inspection of the coupling constants suggested a stereochemically distinct species related to the initially formed penicilloic acid **1** (Figs. 2 and 3). Based on the observed J values and by HRMS and HPLC-MS data (see later), this species was assigned to compound **2** (Figs. 2 and 3), a diastereomeric form arising from the structural rearrangement consistent with epimerization at C6. Although the existence of such species has been proposed for 6-APA,[6, 9] it has never been structurally characterized by NMR nor reported under acidic degradation conditions. The absence of detailed NMR studies in previous reports likely explains why this diastereomer has not been previously identified during acid degradation of 6-APA.

At an acid concentration of 0.75 M, 6-APA undergoes a slow and incomplete degradation over the experimental time window, retaining a significant portion of starting material (Figs. 2 and 4 and Table SI2). The percentage of starting material decreases from approximately 97% at time zero to about 77% after 60 min and remains at around 35% after 330 min. During this period, compound **1** progressively accumulates to about 37%, while compound **2** remains below 20%. At 1.0 M, the hydrolysis becomes faster leading to near-complete consumption within the experimental window: the percentage of 6-APA decreases from 95% at time zero to 54% after 60 min and to 22% after 240 min. Under these conditions, compound **1** becomes the predominant species (45%) while compound **2** increases to approximately 25–28%. At 1.5 M, the degradation is rapid with almost complete disappearance of 6-APA within 120–150 min. Indeed, 6-APA concentration decreases to approximately 21% after only 60 min and to <10% within 120 min. At the same time, compound **1** reaches values above 60% while compound **2** increases to 30–40% at longer reaction times (240 min). At the highest concentration tested (2.0 and 2.5 M), 6-APA disappears almost completely in 30–60 min, demonstrating a pronounced acceleration of the apparent degradation rate.

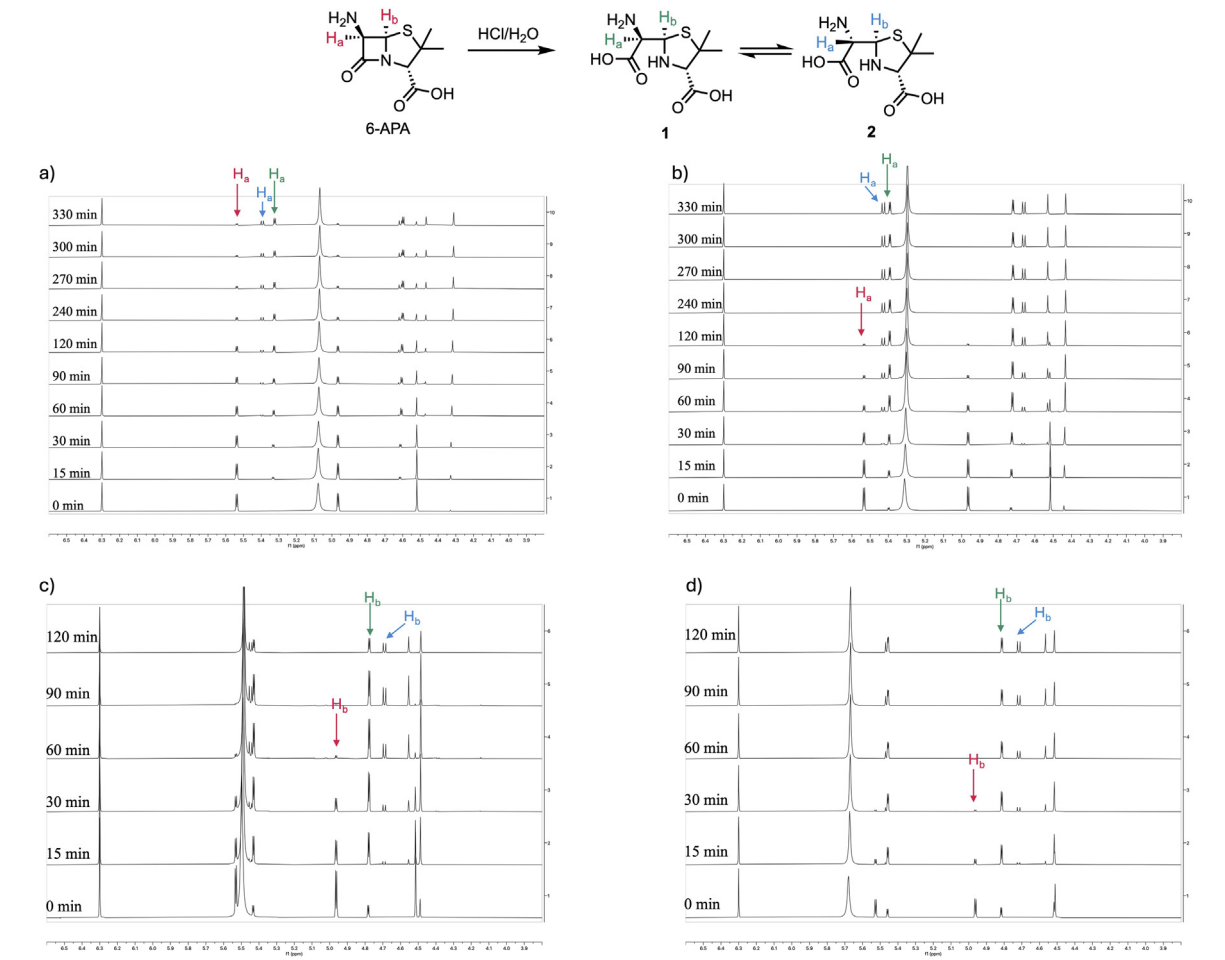

Fig. 3. Section of the time-dependent ^{1}H NMR of 6-APA at different acidic concentration recorded at 25 °C; a) 1.0 M of 6-APA in DCl/D_2O recorded at different time points; b) 1.5 M of 6-APA in DCl/D_2O recorded at different time points; c) 2.0 M of 6-APA in DCl/D_2O recorded at different time points; d) 2.5 M of 6-APA in DCl/D_2O recorded at different time points. Diagnostic resonances used for assignment and kinetic analysis are color-coded: 6-APA (red), compound 1 (green), and compound 2 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

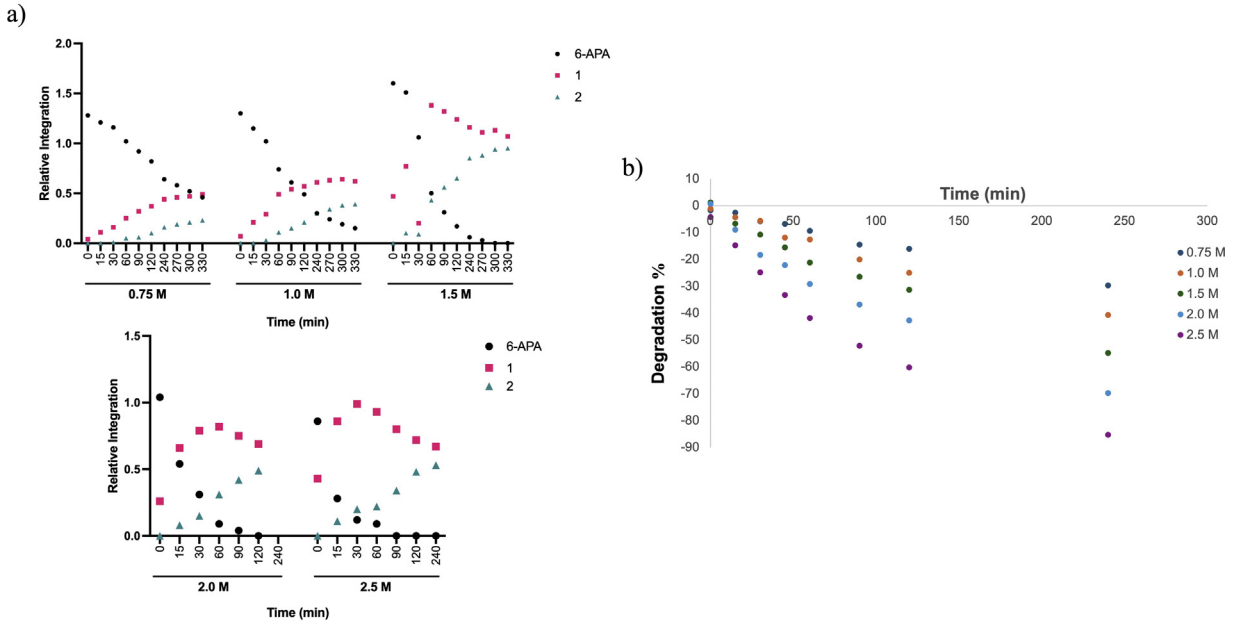

Fig. 4. a) Plots of ^{1}H NMR relative integral intensities versus time monitoring the hydrolysis at 25 °C of 6-APA at different acid concentration (0.75, 1.0, 1.5, 2.0 and 2.5 M); b) time-dependent degradation of 6-APA monitored by HPLC at different acid concentrations. Data are reported as percentage change of the peak area relative to the initial value.

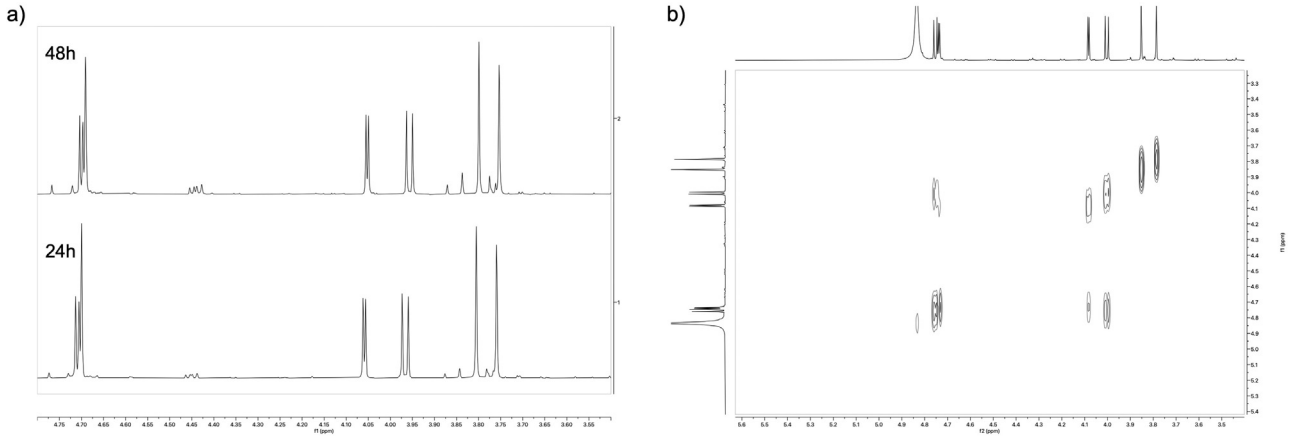

Fig. 5. a) Section of the ^{1}H NMR spectra of a solution of 2.5 M of 6-APA in DCl/D_2O at room temperature recorded at 24 and 48 h. It is clear the presence of two diastereomeric species; b) section of the COSY ^{1}H NMR of 2.5 M of 6-APA in DCl/D_2O recorded at 7 h.

At 2.0 M, percentage of 6-APA falls below 10% after just 60 min and becomes nearly undetectable at later times. At 2.5 M, similar values are reached within 30−60 min. Under these conditions, compound **1** is initially dominant (up to 65−70%), then converting in compound **2** that increases significantly at longer times (240 min), reaching values comparable or higher than 40%.

The trend observed in the ^{1}H—NMR spectra is further confirmed by HPLC analyses conducted in parallel at different acid concentrations. The chromatograms show a significant reduction in the 6-APA signal as acidity increases (see SI) in agreement with the degradation profiles observed by NMR.

Analysis of the time-resolved ^{1}H NMR data shows that the appearance of the new signals becomes progressively faster at higher acid concentration, confirming that β-lactam hydrolysis is strongly promoted by the acidic conditions. These observations are consistent with the proton-assisted mechanism in which protonation of the carbonyl of the β-lactam enhances its electrophilicity, thereby facilitating the nucleophilic attack by water and the cleavage of the strained four-member ring. As reported in the plots of ^{1}H NMR relative integral intensities versus time at different acid concentration (Fig. 4), compound **1** exhibits a kinetic profile characteristic of a reactive intermediate in a sequential transformation. At low acidity (0.75 M), compound **1** accumulates gradually and reaches a stable plateau while at 1.0 M it reaches a higher maximum concentration more rapidly, consistently with increased β-lactam activation. At higher acidic concentrations (2.0 and 2.5 M), a markedly different behaviour is observed. In these conditions, compound **1** shows a pronounced rise to maximum concentration followed by a progressive decrease as the reaction proceed. Such *bell-shaped* profiles are characteristic of intermediates in consecutive A→ B ⇌ C transformations and confirm that **1** is not a terminal degradation product but a transient species. The formation of product **2** shows a complementary trend. At 0.75 M compound **2** forms slowly and to a limited extent. At 1.0−1.5 M, its accumulation becomes evident, particularly at higher times. At 2.0 and 2.5 M, it appears at earlier times and accumulates rapidly.

This behaviour is further confirmed by the ^{1}H NMR spectra recorded for solutions of 6-APA in 2.5 M DCl/D_2O after 24 h, in which an equilibrium between the two species, corresponding to compound **1** and **2**, in solution is clearly observed (Fig. 5a). ^{1}H NMR COSY spectrum further supports the assignment of the signals (Fig. 5b). Under these strongly acidic conditions, the relative integrals of compounds **1** and **2** remain constant over time, indicating that a thermodynamic equilibrium between the two species has been established, and their relative stability up to 48 h.

Literature reports a classic degradation pathway for 6-APA in aqueous solution where β-lactam ring-opening product generate penicilloic acid (compound **1**) through hydrolysis of the amide in solution (Fig. 6a).[5, 6, 8] Sequential decarboxylation of **1** gives compound **3**. Under our experimental condition, however, the decarboxylated product **3** is detected only in trace amount and only after prolonged reaction time (see SI). This observation is confirmed by HRMS analysis of 6-APA solution (DCl/D_2O 2.5 M) after 24 h exposure (Fig. 6b), which clearly shows signals attributable to compounds **1**−**2** and related fragments rather than the decarboxylated species **3**. Consistently, HPLC-MS analysis of the 1.5 M solution of 6-APA reveals two main species formed (RT 6.5 min and RT 6.7 min) corresponding to compounds **1** and **2** (m/z^+ = 235, m/z^- = 189) while a third compound, consistent with the decarboxylated compound **3**, is detected only after 24 h at very low concentration (RT 6.25 min) (see SI).

Taken together, these data suggest that under the investigated conditions the degradation of 6-APA proceeds mainly through the ring opening to form compound **1** which undergoes further structural rearrangements/epimerization, establishing an equilibrium between diastereomeric species **1**−**2** (Fig. 6a).

The kinetics of the reaction were further analysed using time-resolved ^{1}H NMR data. Kinetic profiles were derived from normalized ^{1}H NMR integrals recorded under identical experimental conditions. For each acid concentration the reaction was monitored in the same NMR tube using identical acquisition parameters and the same amount of 6-APA. Maleic acid was used as an internal standard to normalize signal intensities. The disappearance of 6-APA follows pseudo-first-order kinetics at each acid concentration (0.75−2.5 M), as demonstrated by the linear $\ln(C/C_0)$ versus time (Fig. 6c). k_obs values were obtained from the initial linear region of the $\ln(C/C_0)$ vs time plots, where deviations from linearity due to secondary processes are minimal. Deviations from linearity, particularly at lower acid concentrations and longer reaction times, are attributed to secondary processes, including the formation and reversible interconversion of degradation products. The observed pseudo-first-order rate constants (k_obs), obtained from the slope of these plots, increase markedly with increasing acid concentration. The k_obs values obtained for each acid concentration are summarized in Table 1. A log−log plot of ln(k_obs) versus ln([H^+]) shows a clear dependence of the reaction rate on proton concentration, consistent with an acid-catalyzed degradation mechanism. This strong acid dependence indicates that proton activity directly governs the rate-determining activation of the strained β-lactam system. Based on the time-resolved NMR data, the degradation process is better described as an initial acid-catalyzed hydrolysis of 6-APA to compound **1**, followed by a reversible transformation leading to an equilibrium between compounds **1** and **2** (6-APA → 1 ⇌ 2), rather than a simple irreversible A → B → C sequence. While the pseudo-first-order treatment adequately describes the disappearance of 6-APA under conditions of excess acid, a full kinetic model including all species would require

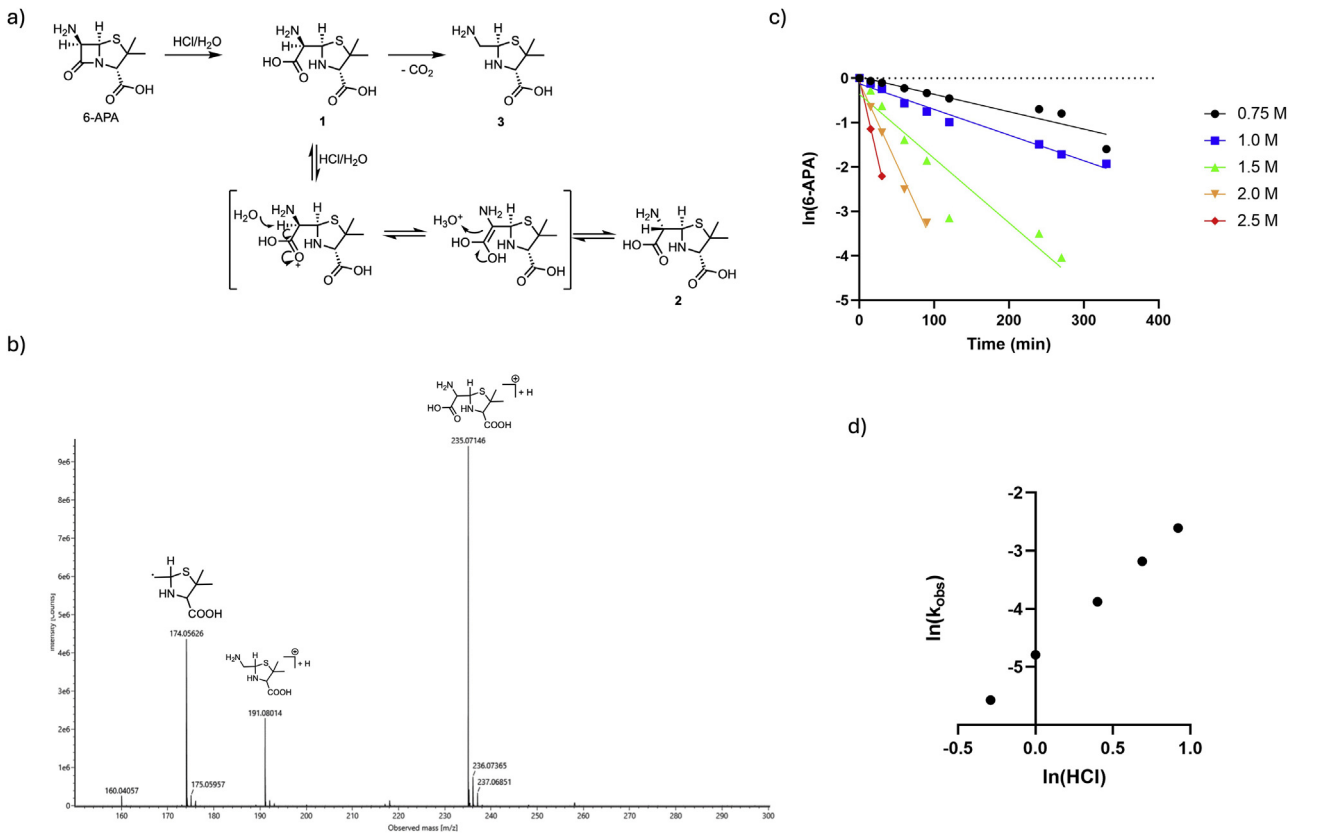

Fig. 6. a) Proposed mechanism for the acid-catalysed transformation of 6-APA in 1 and 2; compound 3 was not detected under our experimental conditions; b) High-resolution Mass Spectrometry of 2.5 M 6-APA in HCl/H_2O after 24 h with assignment of the main observed ions; c) Time-dependent pseudo-first order plots for the acid-catalyzed degradation of 6-APA at 25 °C. The natural logarithm of the normalized 6-APA concentration is plotted as a function of time at different acid concentrations; d) Relationship between the observed k_obs and acid concentration for the degradation of 6-APA at 25 °C. The natural logarithm of k_obs is plotted versus the natural logarithm of HCl concentration.

Table 1
Pseudo-first-order kinetic parameters for the acid-induced degradation of 6-APA obtained from time-resolved ^{1}H NMR experiments.

Concentrations (M)	k_obs (min^{-1})	ln(k_obs)	$t_{1/2}$ (min)	R^2
0.75	0.003917	−5.542	176.9368	0.894405
1.0	0.005778	−5.154	119.9705	0.977884
1.5	0.014478	−4.235	47.87713	0.905554
2.0	0.036779	−3.302	18.84651	0.989829
2.5	0.073628	−2.609	9.414125	0.999529

independent quantification of compounds **1** and **2**, which was beyond the scope of the present study.

Mechanistically, the protonation increases the electrophilicity of the β-lactam carbonyl, thereby facilitating nucleophilic attack by water and promoting ring opening to generate intermediate **1** (penicilloic acid). Subsequently, compound **2** progressively accumulates as [**2**]/[**1**] ratio increases with both time and acid concentration (see SI), indicating that **2** arises from structural reorganization of **1** rather than a parallel pathway originating directly from 6-APA. At higher concentrations (2.5 M), the ^{1}H NMR spectra recorded at 24 and 48 h show no further change in relative integrals of compound **1** and **2**, demonstrating that a thermodynamic equilibrium between the two species has been established. In contrast, the decarboxylated species **3**, reported in literature as a possible downstream product of **1**, is detected by HPLC only in trace and only after longer reaction times (≥ 24 h). This observation indicates that decarboxylation is not a kinetically relevant pathway under these experimental conditions.

Conclusions

Overall, the data support a degradation pathway in which 6-APA undergoes rapid, acid-catalyzed hydrolysis to penicilloic acid (**1**), followed by epimerization to its C6 diastereomer (**2**). Under strongly acidic conditions, these species establish a dynamic equilibrium, while alternative pathways, such as decarboxylation, remain negligible.

Although related species have been proposed under alkaline conditions,[9] while preparing this manuscript, Wu et al. reported the formation of multiple products during the degradation of 6-APA, including evidence for the presence of two diastereomeric species.[7] However, these species were not unambiguously structurally assigned. The present study provides direct NMR-based evidence enabling the identification of one of these species as a C6 epimer of penicilloic acid formed under acidic conditions.[7]

The combined use of time-resolved NMR, HPLC and HRMS provides a clear picture that differs from classical descriptions, highlighting the importance of stereochemical transformations in β-lactam chemistry. These findings have direct implications for controlling the stability of 6-APA in industrial processes and emphasize the need to carefully manage acidic conditions during manufacturing and formulation.[10, 11]

Availability of data and materials

The research data used to support the conclusion can be obtained from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

AM dedicates this manuscript to the Professor Anna Minarini on the occasion of her retirement. This study has been carried out with the contribution of the Alma Mater Studiorum - University of Bologna (RFO funding for fundamental research). This study is the result of a collaborative project between Suanfarma Italia SPA and Alma Mater Studiorum- University of Bologna.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2026.104361.

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