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STUDY ON THE RATE OF EPIMERIZATION OF AMOXICILLIN β-PENICILLOIC ACID TO ITS α-FORM IN AQUEOUS SOLUTIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using automated high-performance liquid chromatography, a preliminary study on the rate of conversion of β-penicilloic acid to its α-form in aqueous solutions, using amoxicillin penicilloic acid as an example is described. An octadecyl-silica column eluted with 0.05 M potassium phosphate buffer solution (pH 5.9) with detection at 228 nm was used for this study. The conversion rate was found to follow pseudo first-order kinetics.

INTRODUCTION

The quantitative determination of antibiotics and their degradation products and impurities is one of the most difficult problems in pharmaceutical analysis. Bioassays and chemical assays of antibiotics are the most frequently used techniques for potency determination. With regard to penicillins, in general, the Code of Federal Regulations[1] states that the potency results obtained from the microbiological assay shall be conclusive. But these bioassay methods have a number of limitations, are non-specific and usually cannot be used to monitor low levels of degradation products and impurities.

On the other hand, research on the possible causes of penicillin allergy has considered four factors which may be of importance: (a) direct reactions of penicillin with proteins *in vivo*, (b) degradation products of penicillin that can react with proteins, (c) impurities other than degradation products that may be present in penicillin prior to formulation into dosage forms and (d) metabolites of penicillin formed *in vivo* subsequent to administration that can react with protein[2,3].

With the advancement of chromatographic techniques, especially high-performance liquid chromatography (HPLC), the identities and levels of these degradation products and impurities can be monitored efficiently and selectively. Being aware of the usefulness of liquid chromatography, several investigators studied the separation of penicillins from their degradation products in various media[3–12].

Various schemes of the degradation of pencillins have been reported and reviewed[2,3,13–15]. It has been proposed that penicilloic acid (PA) was the only primary degradation product of penicillin in basic solutions, though the PA formed can un-

dergo decarboxylation under acidic conditions to yield the corresponding penilloic acid and/or a fluorophore[16].

Questions remain, however, as to the exact conformation of the PA formed. Scheme A details the hydrolysis of amoxicillin (I), with its three asymmetric centers, to the corresponding penicilloic acid (II). Although 8 diastereomers are theoretically possible, the following discussion will center on the asymmetric center at C-6.
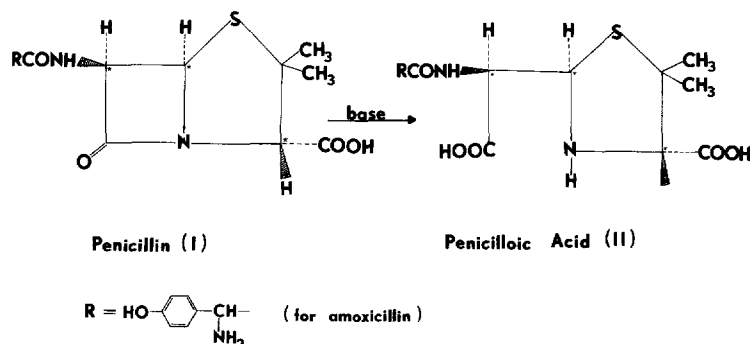
Scheme A. Structures of penicillin (I) and penicilloic acid (II).

Theoretically the PA of a given penicillin can exist as one or all of the four possible stereoisomeric forms and are arbitrarily designed as α, β, γ and δ (see Scheme B)[2,17]. A literature survey showed that in most instances only the term penicilloic acid (e.g. ref. 15) was used.
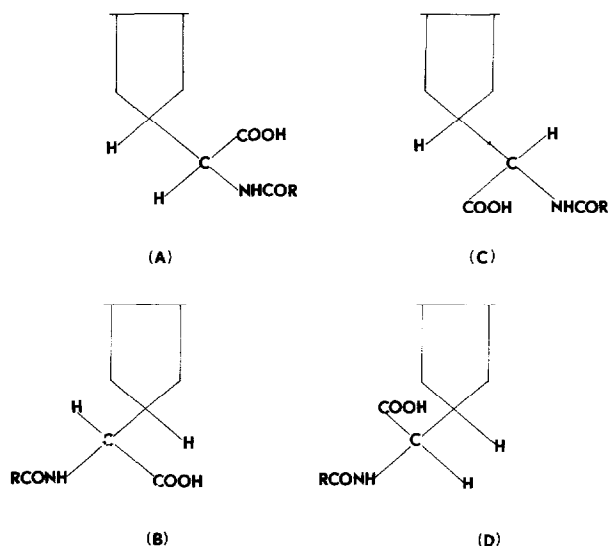
Scheme B. Structures of the four stereoisomeric forms of amoxicillin penicilloic acid. (A) α-PA, (B) β-PA, (C) γ-PA and (D) δ-PA (see scheme A for structure of R group). (Reproduced by permission of Oxford University Press.)

Two recent papers on the HPLC determination of amoxicillin in urine[12,18] showed that there was only one peak for amoxicillin penicilloic acid.

It was not until recently that the terms, α and β forms of PA, were used to describe the two peaks present in the fermentation of penicillin V[9]. Similarly, two peaks corresponding to the penicilloic acid epimers of penicillin G were reported[19,20]. But, so far, nothing was said about the stability of the α and β forms of PA.

In the course of our study on amoxicillin stability, one interesting observation was made. The so-called β-PA, prepared according to a published procedure[15], changed to α-PA in aqueous solution. For discussion purposes, it is assumed that β-PA forms α-PA and not any other stereoisomers. This paper reports the conversion rates of β-PA to α-PA in aqueous solutions using reversed-phase HPLC.

EXPERIMENTAL

Instrumentation

An automated HPLC system used in this study was assembled with the following modular components: a reciprocating piston pump (Model 740-B, Spectra-Physics, Santa Clara, CA, U.S.A.), an auto-injection system (Model 725, Micromeritics, Norcross, GA, U.S.A.), a variable wavelength UV detector (Model SF-770, Schoeffel Spectroflow, Westwood, NJ, U.S.A.; or Model LC-75, Perkin-Elmer, Norwalk, CT, U.S.A.), and an integration system (Model SP-4000, Spectra-Physics).

Reagents and materials

Reagent grade monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) were obtained from Fisher Scientific (Rochester, NY, U.S.A.). Spherisorb-ODS (5 μm) bulk packing material was purchased from Phase Separations (Hauppauge, NY, U.S.A.). An empty stainless-steel column, 316 grade, was obtained from Alltech (Arlington Heights, IL, U.S.A.) and 0.5-μm filters were obtained from Millipore (Bedford, MA, U.S.A.). Amoxicillin penicilloic acid (β-form) was prepared according to a published procedure[15].

Preparations

Column. Spherisorb®-ODS (5 μm) columns (150 × 4 mm I.D.) were slurry-packed in-house as described previously[21].

Mobile phase. One liter of 0.05 M phosphate buffer solution (pH $\approx$ 5.9) was prepared as follows: 10 ml of 0.5 M K_2HPO_4 (stock solution) and 90 ml of 0.5 M KH_2PO_4 (stock solution) were measured into a 1000-ml volumetric flask, which was then filled with distilled water to the mark. This was thoroughly mixed and degassed in an ultrasonic bath (Branson). Notice that either the 0.5 M stock solutions or the prepared mobile phase should be filtered through a 0.5 μm filter before use.

Amoxicillin penicilloic acid solutions. About 15 mg of amoxicillin β-penicilloic acid was accurately weighed into a 25-ml folumetric flask. This was dissolved and diluted to the mark with distilled water. Similarly, amoxicillin β-penicilloic acid in 0.05 M phosphate buffer solution (pH = 5.4) was prepared. Amoxicillin β-penicilloic acid solutions of different concentrations and pH, when needed, were also prepared in similar manner.
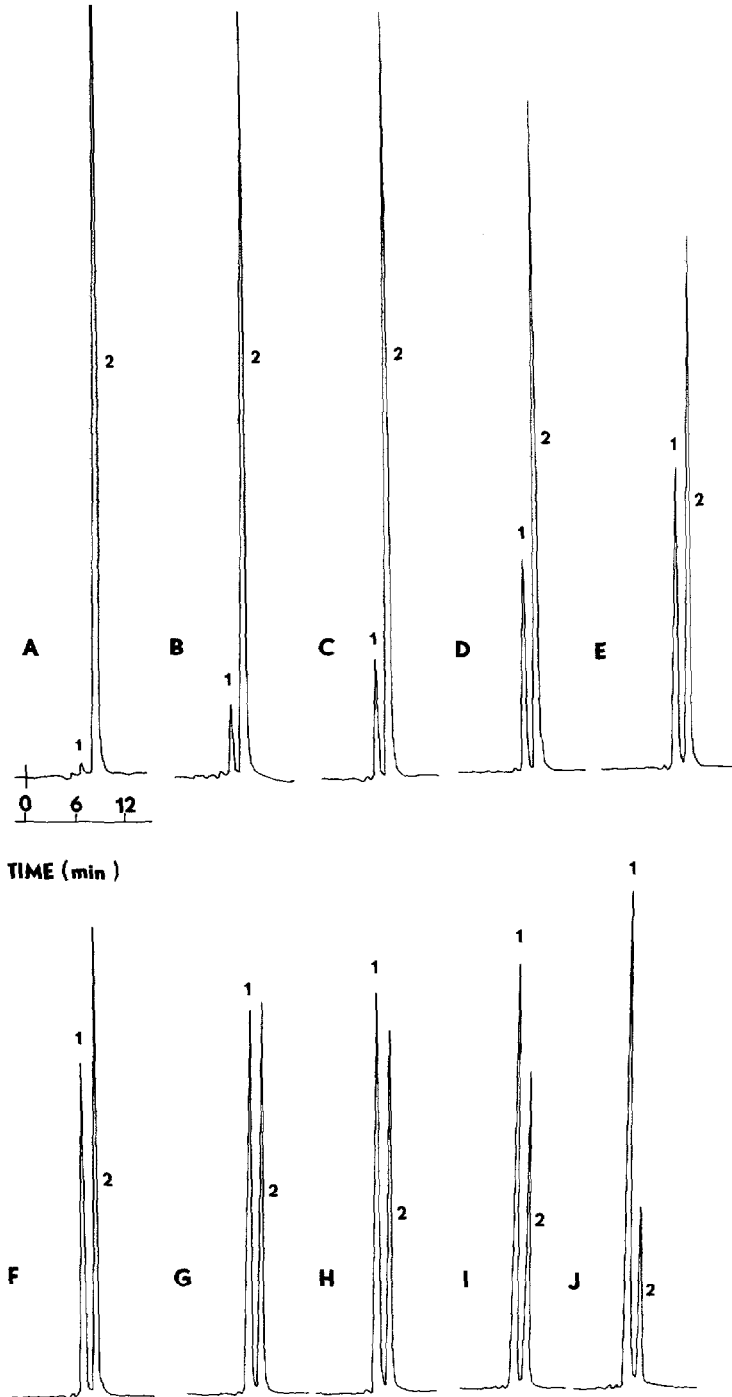
Fig. 1. HPLC scans of amoxicillin penicilloic acids in 0.05 M phosphate buffer (pH 5.4) at room temperature. Conditions: two 150 × 4 mm I.D. Spherisorb® ODS (5 μm) columns in series; mobile phase: 0.05 M phosphate buffer (pH ≈ 5.9); flow-rate: 0.8 ml/min; detection: 228 nm, 0.04 a.u.f.s. Peak identification: 1 = amoxicillin penicilloic acid (α-form), 2 = amoxicillin penicilloic acid (β-form). Time in solution. A = 0.15 h; B = 1.07 h; C = 1.95 h; D = 3.98 h; E = 7.01 h; F = 9.04 h; G = 12.07 h; H = 14.09 h; I = 17.13 h, and J = 31.29 h.

Procedure

The automated HPLC system was set up with detection at 228 nm. Auto-injection of the amoxicillin penicilloic acid solutions (50 μl) at any appropriate time interval up to 99 min per injection could be set for continuous overnight operation. Peak areas were integrated and printed at the end of each assay, allowing 2 min for reporting.

RESULTS AND DISCUSSION

Fig. 1 shows a series of HPLC scans of amoxicillin penicilloic acids (α- and β-forms) in 0.05 *M* phosphate buffer (pH 5.4) at room temperature as a function of time. It is obvious from the first scan (A) that the amoxicillin penicilloic acid, when freshly prepared, was essentially in the β-form. Once in solution, β-PA started converting immediately to the α-form, as evidenced by the series of scans (A to J).

It was noted that, within experimental error, the total peak areas of α- and β-PA were unchanged during the entire assay period.

Similarly, β-PA in 0.05 *M* phosphate buffer at different pHs converted to the α-form but at different rates. Noticeably, the conversion rate was faster at lower pH and slower at higher pH. On the other hand, when β-PA was studied in distilled water (pH ≈ 6.0), the conversion rate was much slower than expected. The accelerated epimerization, or change in configuration at the asymmetric C-6 position, may be the result of catalysis by the phosphate buffer[22]. It was observed that sucrose accelerated the rate of degradation of pencillins in aqueous solution[23].

In the case of the amoxicillin β-PA in pH 5.9 phosphate buffer solution, the amount of β-PA left after 110 h was found to be approximately 13 %. An equilibrium appeared to be reached at this composition because beyond the 110th h, no further
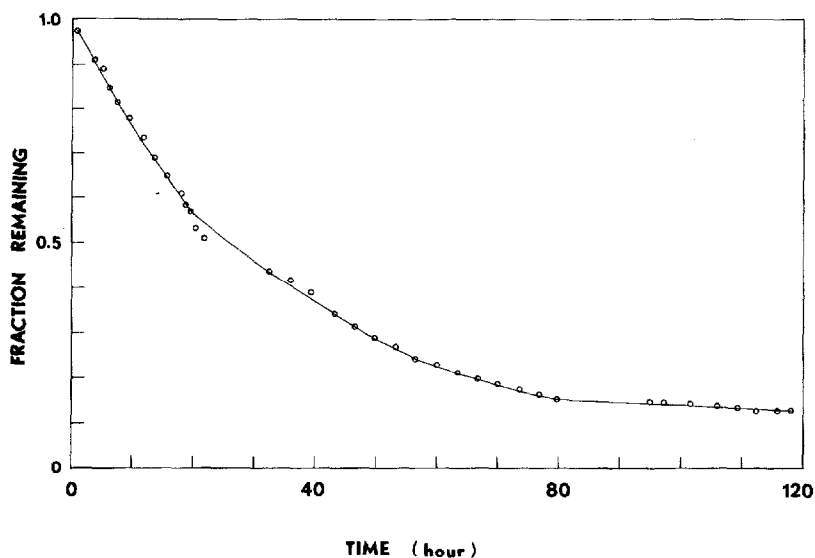

Fig. 2. Plot of fraction remaining of amoxicillin β-penicilloic acid as a function of time (up to ≈ 120 h). Medium: 0.05 *M* phosphate buffer (pH 5.9).
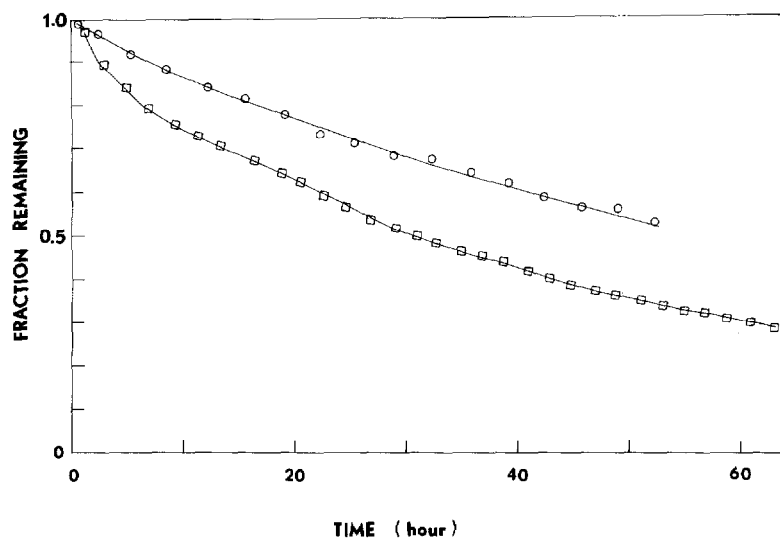
Fig. 3. Plots of fraction remaining of amoxicillin β-penicilloic acid as a function of time (up to $\approx$ 60 h).
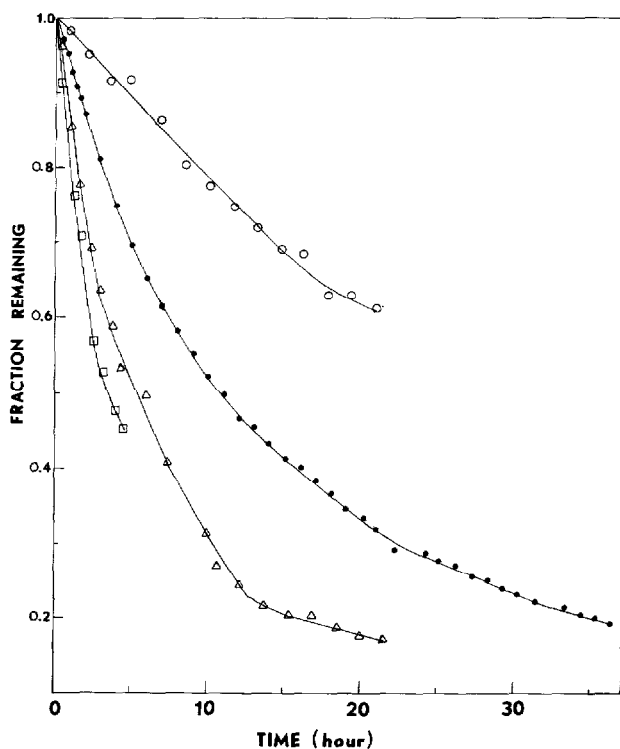Curve identification: ($\bigcirc$), distilled water; ($\square$), 0.05 M phosphate buffer (pH 7.5).

Fig. 4. Plots of fraction remaining of amoxicillin β-penicilloic acid in acidic phosphate buffer solutions
(0.05 M) as a function of time. Curve identification: ($\bigcirc$) pH 6.6; ($\bullet$) pH 5.4; ($\triangle$) pH 4.8 and ($\square$) pH 3.5.
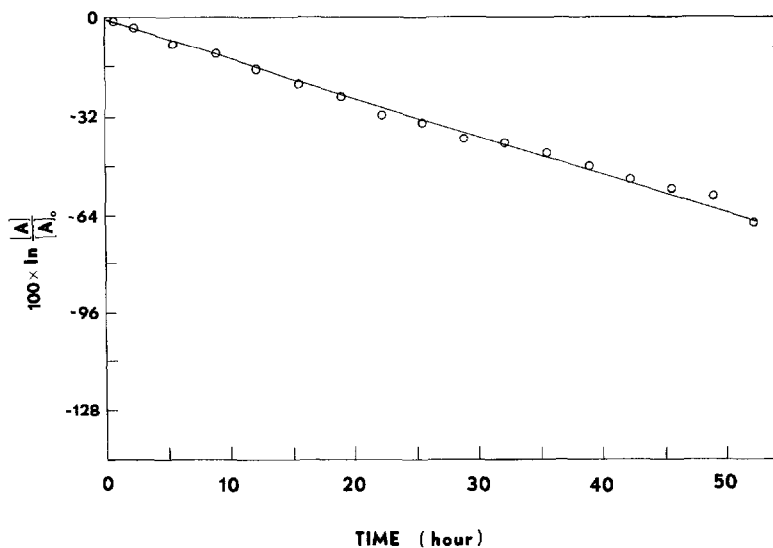
Fig. 5. Linear plot of ln $[A]/[A]_0$ *versus* time for amoxicillin β-penicilloic acid in distilled water.

changes in the ratio of β-PA to its α-form was observed to take place. Indeed, no significant change took place after the 80th h at pH 5.9 (see Fig. 2).

The changes of β-PA with time in water and in various 0.05 M phosphate buffer solutions are depicted in Figs. 3 and 4. The changes resemble that of a typical first-order kinetic reaction and are a function of the nature of the medium and its pH. From the equation for first-order kinetics:

$$\ln \frac{[A]}{[A]_0} = -kt \tag{1}$$

TABLE I

VALUES OF CALCULATED RATE CONSTANTS (k) OF AMOXICILLIN β-PA IN 0.05 M PHOS-PHATE BUFFER SOLUTIONS OF VARIOUS pH AND IN DISTILLED WATER

Initial concentration of amoxicillin β-PA (µg/ml)	pH of medium	Calculated rate constant k (h^{-1})*	Calculated half-life τ (h)**
13	3.5	0.2050	3.4
13	4.8	0.1246	5.6
13	5.4	0.0622	11.1
14	5.9	0.0310	22.3
13	6.6	0.0248	28.0
13	7.5	0.0223	31.1
14	9.1	0.0361	19.3
14	12.0	0.0587	11.8
558	Distilled water	0.0133	52.1

  * Based on the equation ln $[A]/[A]_0$ = $-kt$ and from data collected in the first 24 h or less after dissolution of amoxicillin β-PA.
  ** Based on the equation τ = ln $2/k$, where τ is the half-life of amoxicillin β-PA in a given medium.
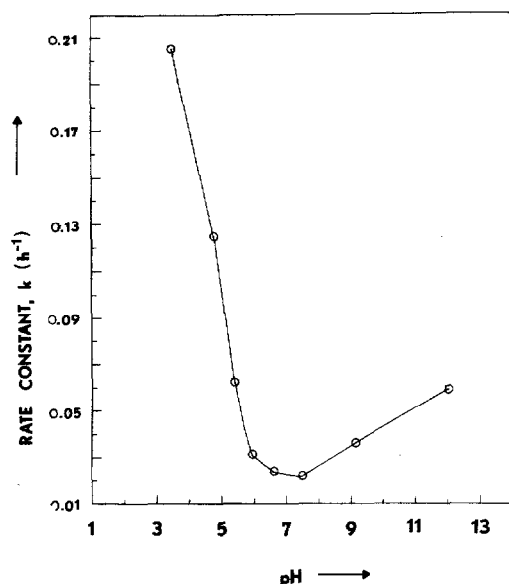
Fig. 6. Plot of rate constant (k) as a function of pH.

where $[A]_0$ = initial concentration of β-PA at time zero; $[A]$ = concentration of β-PA at any time t; k = rate constant; t = time. A plot of the logarithm of the fraction remaining of β-PA against time is shown in Fig. 5. This shows that the conversion rate is first-order with respect to β-PA. The best lines fitting these data were obtained with a linear regression program. The calculated rate constants (k), as obtained from the slopes, are shown in Table I and graphically in Fig. 6. It is obvious, from Fig. 6, that the rate of conversion from the β-form to the α-form was highly favored in acidic and especially alkali media.

TABLE II

COMPARISON BETWEEN THE OBSERVED HALF-LIFE AND CALCULATED HALF-LIFE OF AMOXICILLIN β-PA IN PHOSPHATE BUFFER SOLUTIONS OF VARIOUS pH AND IN DIS-TILLED WATER

pH	Observed half-life $\tau_{obs.}$ (h)	Calculated half-life τ_{cal} (h)*	Correlation coefficient (γ)*
3.5	3.5	3.4	−0.9923
4.8	5.6	5.6	−0.9812
5.4	11.1	11.0	−0.9976
5.9	22.4	22.3	−0.9967
6.6	N.A.**	28.0	−0.9952
7.5	30.7	31.1	−0.9783
9.1	19.5	19.3	−0.9994
12.9	11.8	11.8	−0.9989
Distilled water	N.A.**	52.1	−0.9997

  * Based on the plots of ln $[A]/[A]_0$ versus t.
 ** N.A. = not available.

Value for the half-life (τ) of β-PA in various aqueous solutions were calculated using the equation, $\tau = \ln 2/k$, for first-order reactions (Table I). A comparison between the observed and calculated half-lives is shown in Table II. The large negative correlation coefficients (γ ranges from -0.9997 to -0.9783) indicate that the first-order kinetic equation can adequately describe the conversion of β-PA in aqueous solution and that the HPLC technique as used here is an appropriate method to carry out the kinetic analysis.

It was also observed that no peaks corresponding to the α- and β-PA were detected when a strongly acidic solution (*e.g.* 0.1 *M* HCl) of amoxicillin β-PA was assayed under the same conditions. Presumably, complete decarboxylation of amoxicillin β-PA to penilloic acid(s) and further decomposition products takes place rapidly in strongly acidic solution.

ACKNOWLEDGEMENTS

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