Investigation of a Proposed Penicillin G Acidic Degradation Scheme using High-pressure Liquid Chromatography and Optimization Techniques and Mechanistic Considerations

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Optimization techniques were used to fit a recently proposed degradation scheme to recently published n.m.r. data for the time course of penicillin G and four degradation products at pH 2.5 and 37 °C. Several conclusions arising from the n.m.r. analysis which were originally associated with the degradation scheme were not compatible with the optimized rate constants. It was necessary to change substantially the proportion of penicillin G degrading through benzylpenicillenic acid, benzylpenillic acid, and benzylpenicilloic acid in order for the degradation scheme to fit the n.m.r. data. Benzylpenillic acid replaced benzylpenicillenic acid as the major product. The rate constants best describing the n.m.r. data showed benzylpenicillenic acid proceeding almost exclusively through benzylpenamaldic acid. Such optimization implied that the scheme could be simplified to three parallel reaction pathways, the dominant reaction occurring through benzylpenillic acid. However, mechanistic considerations indicate that the direct conversion of penicillin G into benzylpenillic acid is not possible and that a likely intermediate is benzylpenicilloic acid. The degradation of benzylpenicilloic acid at pH 2.5 was consequently monitored by ion-pair reversed-phase high-pressure liquid chromatography and rapid formation of benzylpenillic acid was detected. This observation is inconsistent with the recently proposed degradation scheme, even though the scheme can be made to fit the n.m.r. kinetic data.

The acidic degradation of penicillin G (1) was recently studied by n.m.r.[1] The results were kinetically analysed and Scheme 1 was proposed.[1] An examination of the fit of the theoretical predictions to the experimental data revealed that the concentrations predicted by Scheme 1 did not coincide with the n.m.r. data for penicillin G (1) or any of the degradation products.[2] The purpose of this study was to optimize the fit of Scheme 1 to the n.m.r. data to determine if they were compatible. In carrying out the optimization, the following original kinetic restrictions were ignored: (1) that the rate constant for the loss of penicillin G (1) be 0.44 min^{-1}; (2) that the proportion of penicillin G (1) which degraded through benzylpenicillenic acid (2), benzylpenillic acid (3), and benzylpenicilloic acid (4) be in the ratio of 50 : 37 : 13; (3) that the rate constant for the loss of benzylpenicillenic acid (2) be 0.7 min^{-1}; (4) that the proportion of benzylpenicillenic acid (2) which degrades through benzylpenamaldic acid (5), benzylpenillic acid (3), and benzylpenicilloic acid (4) be in the ratio of 24 : 20 : 6.

Experimental

Benzylpenicilloic acid (4) and benzylpenillic acid (3) were synthesized by standard methods.[3] Benzylpenicilloic acid (4) (0.05M) was dissolved in a 0.1M-phosphate buffer at pH 2.5 and aged at 37 °C. Samples were analysed by reversed-phase h.p.l.c. (ALC 202 and Ultrasphere-ODS; Beckman) utilizing a u.v. detector at 254 nm. The mobile phase was composed of 30% acetonitrite and 70% 0.006M-phosphate buffer at pH 7.5 containing 0.008M-tetrabutylammonium chloride.[4] The flow rate was 1.5 ml min^{-1} and the u.v. attenuation was 0.16 a.u.f.s. (absorbance units full scale).

Results and Discussion

The system of differential equations (1)—(6) which describes Scheme 1 follows, where for example, C_1 denotes the concentration of penicillin G (1). These equations are of a common form and can be integrated analytically one by one. The solution to each equation takes the form of a sum of one or more exponential functions in time t. For example, the simplest is the solution (7) for the first equation where C_1 is the concentration of penicillin G (1) (C_1^0 is the initial concentration).

$$-dC_1/dt = k_aC_1 + k_hC_1 + k_iC_1 \quad (1)$$

$$-dC_2/dt = -k_aC_1 + (k_b + k_c + k_g)C_2 \quad (2)$$

$$-dC_3/dt - -k_iC_1 - k_bC_2 + k_eC_3 \quad (3)$$

$$-dC_4/dt - -k_hC_1 - k_gC_2 + k_jC_4 \quad (4)$$

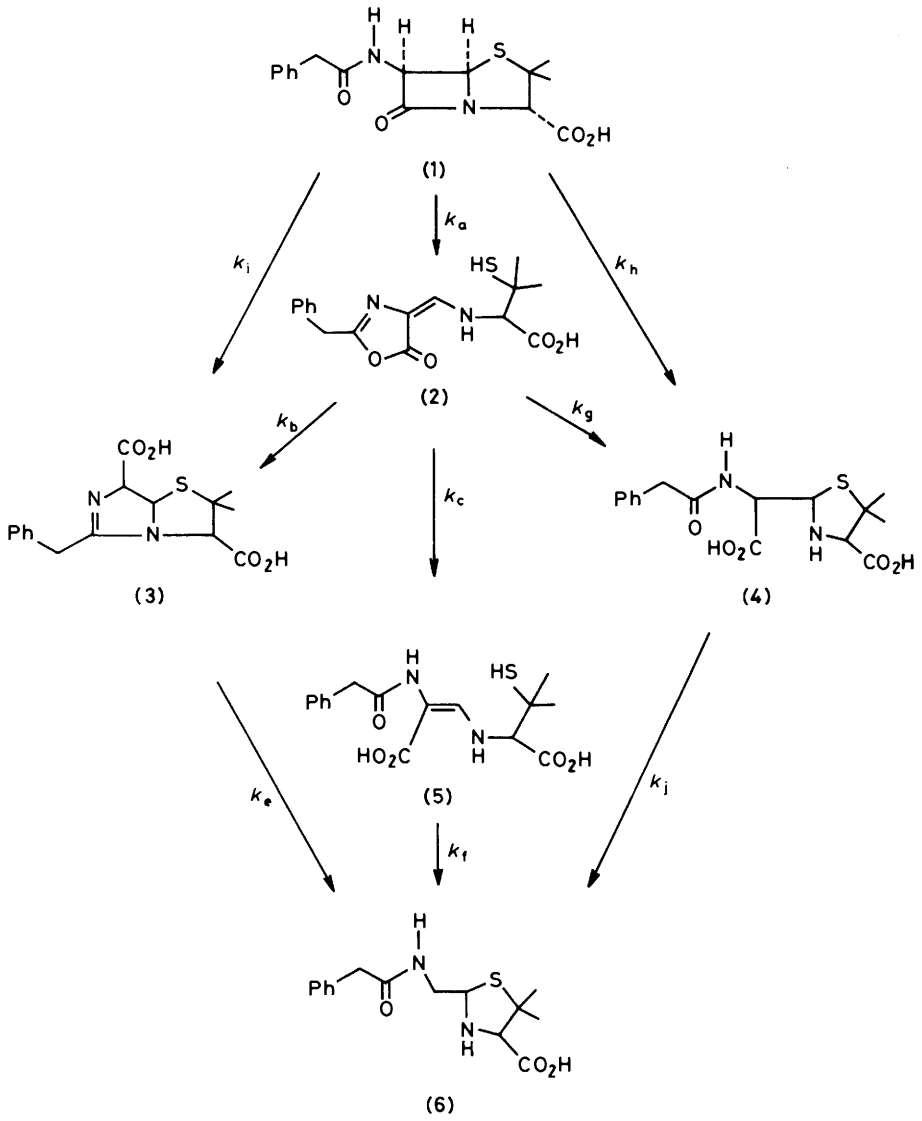
$$-dC_5/dt = -k_eC_2 + k_fC_5 \quad (5)$$

$$-dC_6/dt = -k_eC_3 - k_jC_4 - k_fC_5 \quad (6)$$

$$C_1 = C_1^0e^{-(k_a+k_h+k_i)t} \quad (7)$$

The system of equations was integrated analytically and the solution was programmed for a digital computer. The solution contained a check to ensure that the material balance was always observed after the computation. This solution was used to optimize the values of the rate constants in the theoretical model to obtain the best fit of the model to the experimental data.

It is not at all apparent which criterion to use to obtain the best fit. In fact, the definition of which fit is best depends upon the use to which the model is to be put. For example, if one were interested only in one of the components of the reaction scheme, the best fit would describe the time course of that component most accurately. If one were interested in more than one component, a variety of weightings would be possible depending on the relative importance of error from one component to the next. Since the overall fit of Scheme 1 is the point of interest, all compounds were incorporated into the optimization criterion.



Scheme 1.

Further, the question arises as to whether the deviation should be measured on the same scale for all components or as a proportion of the concentration of that component. This becomes a very significant question when, as in the data analysed here, the range of concentrations is large. Under this circumstance the same deviation in the magnitude will represent a small percentage deviation for a component present in large concentration, but a very great percentage deviation for a component present in small concentrations.

Absolute deviations were used instead of relative or percentage deviations because no criterion was available which suggested that relative deviations were more important. With the small concentrations found for some components, these components would for two reasons have unduly influenced the rate constants obtained had relative deviations been used: (1) proportionately larger analytical error at small concentrations; (2) small absolute error representing very large relative error.

A further question arises regarding whether to use as a criterion of optimality the sum of absolute deviations, the sum of squared deviations, *etc*. The sum of squared deviations was selected for this study.

Any number of optimization schemes could have been used for optimization, and, in fact, more than one was tried. Initially, Gauss–Seidel [5] and gradient methods [6] were tried, but it was found that simple direct search of one variable at a time was most satisfactory.

The optimization procedure was effective in finding a set of rate constants (Table 1), which caused Scheme 1 to fit the n.m.r. data. The original rate constants [1,2] gave a mean square absolute deviation of 2.87 while the optimized rate constants gave a mean square absolute deviation of 0.026. The excellent fit of the n.m.r. data to Scheme 1 with the optimized rate constants is seen clearly in Figures 1 and 2.

Thus, the optimization of the rate constants made Scheme 1 compatible with the n.m.r. data. However, several conclusions,[1] originally associated with Scheme 1, are not compatible with the optimized rate constants. The proportion of penicillin G degrading through benzylpenicillenic acid (2), benzylpenillic acid (3), and benzylpenicilloic acid (4) was originally reported to be 50 : 37 : 13. The relative values of optimized k_a, k_i, and k_h (Table 1) indicate that benzylpenillic acid (3) represents the main product (58%) while benzylpenicillenic acid (2) (23%) and benzylpenicilloic acid (4) (19%) are secondary products of approximately equal importance.

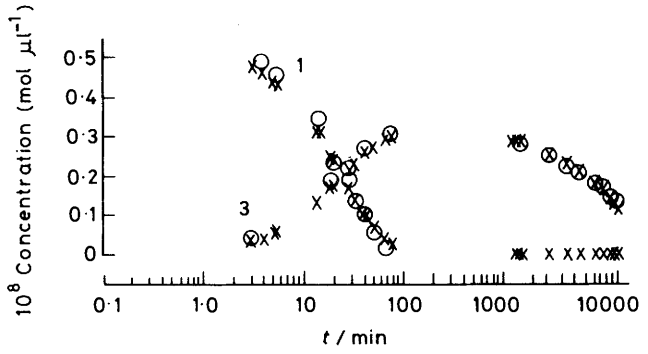


Figure 1. Comparison of n.m.r. analytical concentration of penicillin G (1) and benzylpenillic acid (3), O, to predicted concentration based on optimization Scheme 1, ×

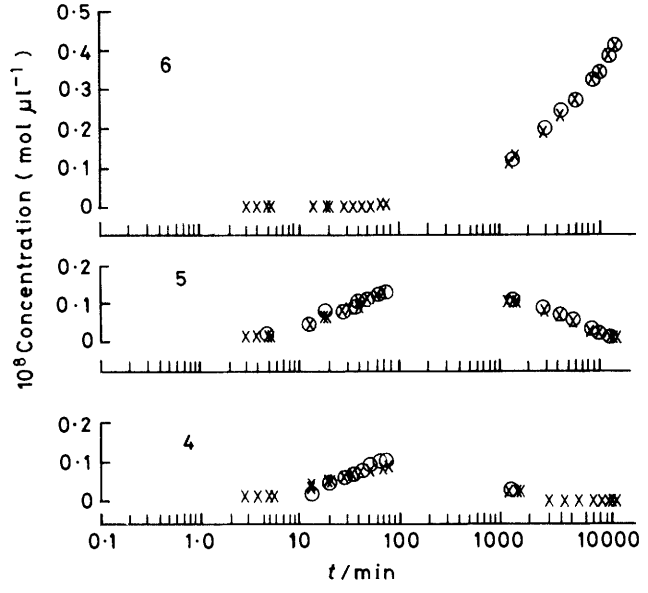
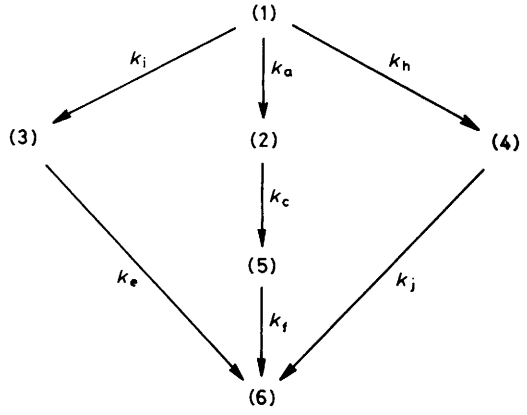


Figure 2. Comparison of n.m.r. analytical concentration of benzylpenicilloic acid (4), benzylpenamaldic acid (5), and benzylpenilloic acid (6), O, to predicted concentration based on optimization Scheme 1, ×



Scheme 2.

In addition, comparison of the optimized rate constants associated with the loss of benzylpenicillenic acid (2), *i.e.* k_c, k_b, and k_g (Table 1) indicate that the pathway through benzylpenamaldic acid (5) is favoured over the routes through benzylpenillic acid (3) or benzylpenicilloic acid (4) by a factor of *ca.* 10^9 and 10^8, respectively. The original Scheme 1 indicated that benzylpenamaldic acid (5), benzylpenicillic acid (3), and benzylpenicilloic acid (4) form from benzylpenicillenic acid (2) in a ratio of 24 : 20 : 6. Thus, the optimized rate constants indicate that, in a practical sense, all the benzylpenicillenic acid (2) formed is transformed into benzylpenamaldic acid (5). As noted in Table 1, the mean square absolute deviation for the optimized scheme did not change when k_b and k_g were set equal to 0.

Table 1. Original [a] and optimized rate constants (min^{-1}) for Scheme 1

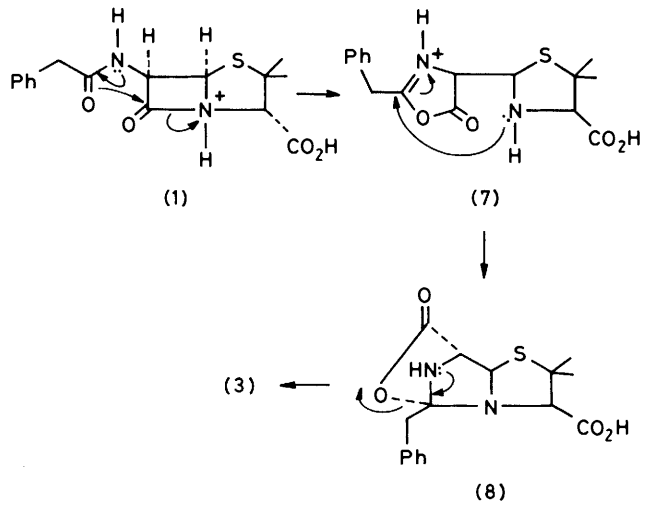
Rate constant	Original [a]	Optimized	Optimized and simplified
k_a	2.2×10^{-1}	9.8×10^{-3}	9.8×10^{-3}
k_b	2.8×10^{-1}	8.9×10^{-8}	0.0
k_c	3.4×10^{-1}	57.1	57.1
k_e	5.7×10^{-5}	6.5×10^{-5}	6.5×10^{-5}
k_f	2.4×10^{-5}	1.5×10^{-4}	1.5×10^{-4}
k_g	8.0×10^{-2}	9.6×10^{-7}	0.0
k_h	6.0×10^{-2}	7.8×10^{-3}	7.8×10^{-3}
k_i	1.6×10^{-1}	2.5×10^{-2}	2.5×10^{-2}
k_j	1.9×10^{-5}	1.0×10^{-3}	1.0×10^{-3}
Mean square absolute deviation	2.87	0.026	0.026

[a] From refs. 1 and 2.

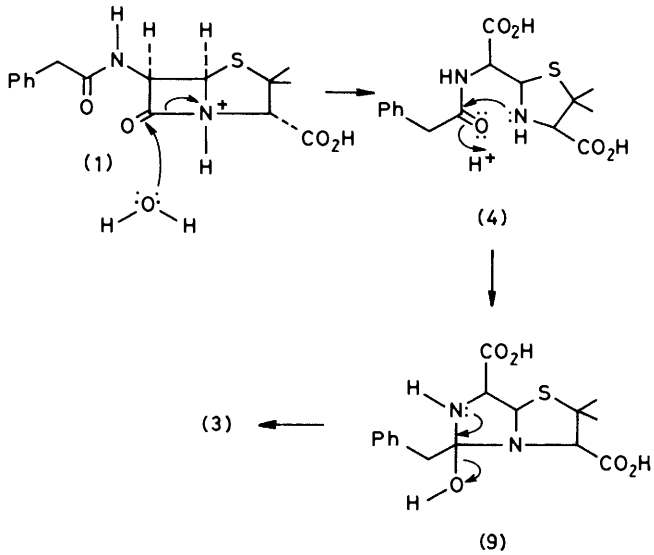
The values of optimized k_b and k_g are so small that Scheme 1 can be simplified by deleting these reactions. Thus, Scheme 2, which contains three parallel reaction pathways with the dominant reaction occurring through benzylpenillic acid (3), can be used as an empirical description of the acidic degradation pathways of penicillin G (1).

The direct conversion of penicillin G (1) into benzylpenillic acid (3) was proposed in Scheme 1 [1] and became the dominant reaction when Scheme 1 was fitted to the n.m.r. data. However, consideration of the possible mechanisms involved in this transformation leads to the conclusion that a direct conversion of penicillin G into benzylpenillic acid is not possible.

Most of the arguments which have appeared in the literature concerning the mechanism of the rearrangement of penicillin G (1) to benzylpenillic acid (3) have been highly influenced by the early suggestion that compound (7) (Scheme 3) is an obligatory intermediate in this transformation.[7] Several pieces of evidence do agree with the pathway portrayed in Scheme 3. In the first place, it has been shown that benzylpenicillins can be rendered more acid stable by the incorporation of electron-withdrawing substituents in the α-position of the amide side-chain. It has been implied that this effect is due to a decrease in the nucleophilicity of the side-chain amide carbonyl, thus reducing the rate of conversion of these penicillins into intermediates corresponding to compound (7) (Scheme 3).[8,9] It is therefore not surprising that all the schemes which have been proposed for benzylpenicillin G (1) degradation under acidic conditions show benzylpenillic acid (3) and benzylpenicilloic acid (4) on completely separate pathways.[1,2,10,11] Secondly, the conversion of intermediate (7) into benzylpenillic acid (3) is supported by the observation that when (±)-benzylpenicillenic acid (2) was allowed to stand in 95% ethanol at room temperature for 24 h, a 25% yield of benzylpenillic acid (3) was obtained.[12] An intramolecular Michael addition of the sulphhydryl group to the αβ-unsaturated carbonyl moiety present in benzylpenicillenic acid (2) would be expected to yield the proposed azlactonethiazolidine intermediate (7).



Scheme 3.



Scheme 4.

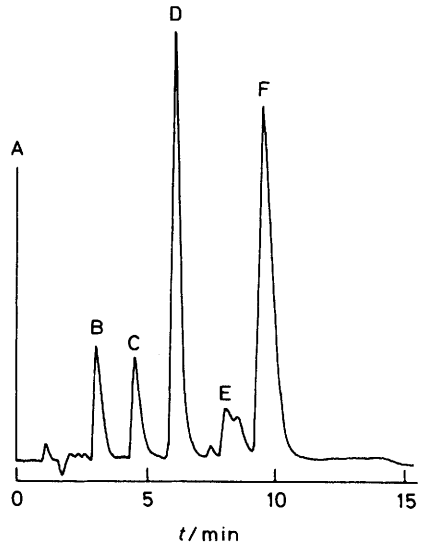


Figure 3. High-pressure liquid chromatogram recorded 1.5 h after benzylpenicilloic acid (4) had been added to the phosphate buffer at 37 °C and pH 2.5. Key: A, time of injection; B, benzylpenicillenic acid (2); C, unidentified compound; D, benzylpenillic acid (3); E, diastereoisomeric benzylpenilloic acids (6); F, benzylpenicilloic acid (4)

Table 2. Ratio of peak heights of benzylpenicilloic acid (4) and benzylpenillic acid (3) during ageing of 0.05M-benzylpenicilloic acid (4) at pH 2.5 and 37 °C

	Peak height (cm)		Ratio of benzylpenicilloic acid (4) to benzylpenillic acid (3)
t/h	Benzylpenicilloic acid (4)	Benzylpenillic acid (3)	
0.00	7.40	0.35	21.14
0.25	7.30	4.10	1.78
0.50	7.35	6.50	1.13
0.75	7.10	7.55	0.94
1.00	7.00	8.10	0.86
1.50	6.80	8.35	0.81
2.00	6.55	8.60	0.76
2.67	6.15	8.80	0.70
3.00	6.15	9.10	0.68
4.00	5.65	8.90	0.63
5.00	5.20	8.95	0.58

We have recently been interested in investigating the alternative possibility that benzylpenillic acid (3) is formed from benzylpenicilloic acid as outlined in Scheme 4. The overall reaction for the conversion of benzylpenicillin G (1) into benzylpenillic acid (3) proposed here conceptually involves three events: (1) an acid-catalysed hydrolysis of the β-lactam bond; (2) a nucleophilic attack by the thiazolidine nitrogen on the protonated amide carbonyl to form a five-membered ring; and (3) the dehydration of the resulting carbinolamine (9) to form the imine bond present in benzylpenillic acid (3).

Direct experimental evidence in support of Scheme 4 was obtained by examining the degradation of benzylpenicilloic acid (4) at pH 2.5. An ion-pair reversed-phase h.p.l.c. method which is capable of separating benzylpenicilloic acid (4) and benzylpenillic acid (3) was employed [4] to monitor the degradation of benzylpenicilloic acid (4) at pH 2.5. As seen in Table 2, benzylpenillic acid (3) formed rapidly from benzylpenicilloic acid (4).

Examination of the chromatogram (Figure 3) indicates that benzylpenillic acid (3) is the major intermediate of benzylpenicilloic acid (4) degradation. This observation is in contrast to the generally accepted view [10,11] that benzylpenilloic acid decarboxylates rapidly to benzylpenilloic acid (6) under acid conditions.

As Scheme 1 provides no pathway for the formation of benzylpenillic acid (3) from benzylpenicilloic (4) it does not describe the acidic degradation of penicillin G even though it can be fitted to the n.m.r. kinetic data.

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