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RESEARCH ARTICLES

Preparation and Immunological Cross-Reactions of Penicilloic and Penilloic Acids

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Received November 22, 1976, from the *Research Division, Beecham Pharmaceuticals, Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey, RH3 7AJ, England.* Accepted for publication December 9, 1977. *Present address: Glasgow and West of Scotland Blood Transfusion Service, At Law Hospital, Carluke, Lanarkshire, Scotland.

Abstract □ Methods are described for the preparation of pure crystalline samples of the penicilloic and penilloic acids of penicillin G, carbenicillin, cloxacillin, floxacillin, methicillin, penicillin V, and ticarcillin and the penicilloic acids of amoxicillin, ampicillin, phenethicillin, and propicillin. The interaction between the compounds and rabbit antibenzylpenicilloyl antibodies was evaluated by hemagglutination inhibition measurements. A significant correlation was found in this system between the reactivity of penicilloic acids and the corresponding penilloic acids; on average, the penicilloic acids were more reactive on a molar basis by a factor of 11. The results are discussed in terms of the general immunochemistry and side-chain structure of the parent penicillins.

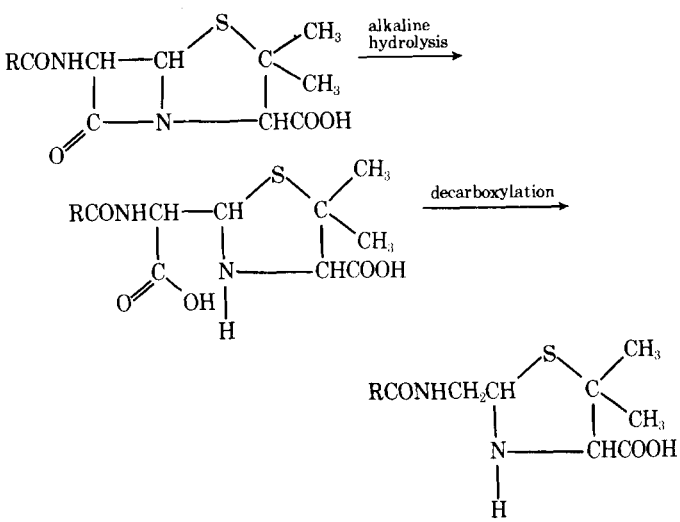
Keyphrases □ Penicilloic acids, various—synthesized, immunological cross-reactions evaluated *in vitro* □ Penilloic acids, various—synthesized, immunological cross-reactions evaluated *in vitro* □ Immunological cross-reactions—various penicilloic and penilloic acids evaluated *in vitro* □ Antibacterials—various penicilloic and penilloic acids, immunological cross-reactions evaluated *in vitro*

The major degradation products of penicillins are the corresponding penicilloic acids, formed by alkaline hydrolysis of the β-lactam ring (Scheme I). The penicilloic acids thus formed can undergo decarboxylation under relatively mild acidic conditions to yield the corresponding penilloic acids.

BACKGROUND

Penicilloic acids were identified as major metabolites of penicillins in humans (1); they have been used, either individually (2) or in combination with the parent penicillins and penilloic acids, as the "minor determinant mixture" (3) to skin test patients suspected of allergy to penicillin. The minor determinant mixture usually comprises the derivatives of penicillin G (benzylpenicillin), but similar mixtures derived from other penicillins also have been used in human skin tests (4–6).

Although it has not been established how the minor determinants combine with proteins *in vivo* to initiate an immune response of the allergic type, the most common reaction occurring between penicillins and proteins involves a nucleophilic amino group on the protein carrier, yielding a penicilloyl group (Structure A) referred to as the "major determinant." Since the penicilloyl determinant group is frequently implicated in immediate hypersensitivity reactions to penicillin, the immunological cross-reactivity between the penicilloyl groups derived from various therapeutic penicillins is of interest. The interaction between



Scheme I

antipenicilloyl antibodies and various penicillins as the penicillins themselves (7), as the derived penicilloic acids (8), or as the penicilloyl derivatives of ε-aminocaproic acid (9) was studied previously.

The preparation of the penicilloic and penilloic acids from penicillin G in pure form was described (2, 10). Detailed methods for the preparation of pure forms of the corresponding materials from other penicillins have been lacking, however, and many *in vitro* and skin test studies utilized chemically uncharacterized materials. The objectives of the present study were to prepare pure samples of the penicilloic and penilloic acids of a number of penicillins and to evaluate their potential to cross-react with benzylpenicilloyl-specific antibodies.

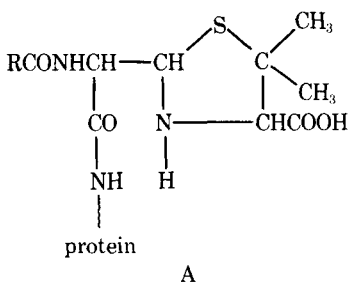


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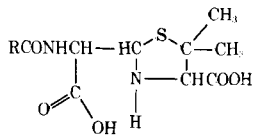
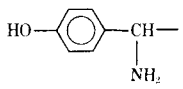
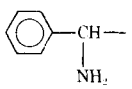
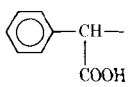
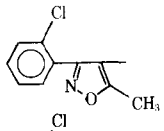
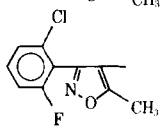
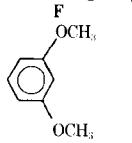
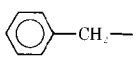
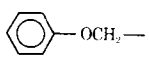
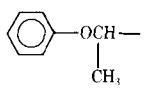
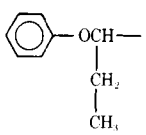
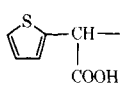
Table I—Penicilloic Acids



Parent Penicillin	R	Formula		Analysis, % Calc.	Found	Equivalent Weight[a] Calc.	Found	Purity[b], %	$[\alpha]_D$[a]	R_f (TLC)
Amoxicillin		$C_{16}H_{20}N_3NaO_6S \cdot H_2O$	C H N Na S	45.39 5.24 9.92 5.43 7.57	45.34 5.25 9.93 5.69 7.11	141.1	145.4[c]	96.1	+103.8° (c 1.0, water)	0.25
Ampicillin		$C_{16}H_{20}N_3NaO_5S \cdot H_2O$	C H N Na S	47.17 5.44 10.31 5.64 7.87	46.79 5.18 10.21 5.74 8.61	135.8	139.1[c]	102.2	+98.1° (c 1.0, water)	0.25
Carbenicillin		$C_{17}H_{19}N_2O_7S \cdot H_2O$	C H N S	49.33 5.02 6.78 7.76	49.77 5.40 6.77 7.96	137.8	138.4[d]	100.3	−15.1° (c 1.0, methanol)	0.20
Cloxacillin		$C_{19}H_{20}ClN_3O_6S \cdot H_2O$	C H Cl N S	48.36 4.70 7.51 8.90 6.79	48.24 4.69 7.28 9.03 6.73	236.0	234.4[d]	109.5	+71.8° (c 1.0, methanol)	0.46
Floxacillin		$C_{19}H_{17}ClFN_3Na_2O_6S \cdot 1\frac{1}{2}H_2O$	C H N Na S	42.04 3.71 7.74 8.47 5.91	41.65 3.54 7.53 8.90 6.25	0	0[d]	106.0	+94.5° (c 1.0, water)	0.39
Methicillin		$C_{17}H_{22}N_2O_7S \cdot H_2O$	C H N S	49.03 5.81 6.73 7.70	49.15 5.85 6.71 7.77	208.2	203.8[d]	97.7	+81.2° (c 1.0, methanol)	0.34
Penicillin G		$C_{16}H_{19}N_2NaO_5S \cdot H_2O$	C H N Na S	48.97 5.39 7.14 5.86 8.17	49.09 5.27 7.23 6.01 8.44	392.4	390.5[d]	95.6	+157.1° (c 1.0, water)	0.34
Penicillin V		$C_{16}H_{20}N_2O_6S \cdot H_2O$	C H N S	49.73 5.74 7.25 8.30	49.77 5.74 7.08 8.10	193.2	193.9[d]	94.8	+95.6° (c 1.0, methanol)	0.40
Phenethicillin		$C_{17}H_{22}N_2O_6S \cdot H_2O$	C H N S	50.09 6.04 7.00 8.01	50.69 5.95 6.95 7.98	200.2	203.2[d]	99.7	+88.6° (c 1.0, methanol)	0.43
Propicillin		$C_{18}H_{24}N_2O_6S \cdot 1\frac{1}{2}H_2O$	C H N S	51.05 6.43 6.61 7.57	51.03 6.48 6.57 7.20	211.7	210.7[d]	100.4	+67.9° (c 1.0, methanol)	0.46
Ticarcillin		$C_{15}H_{18}N_2O_7S_2 \cdot \frac{1}{2}H_2O$	C H N S	43.79 4.65 6.81 15.59	44.01 4.60 6.85 16.00	137.2	133.5[d]	100.2	−22.3° (c 1.0, water + sodium hydroxide → pH 7.0)	0.21

[a] Corrected for water. [b] From iodometric assay. [c] By nonaqueous titration. [d] By aqueous titration.

EXPERIMENTAL

Materials—The following materials were used: amoxicillin [6-[(−)-α-amino-4-hydroxyphenylacetamido]penicillanic acid trihydrate], ampicillin [6-[D-(−)-α-aminophenylacetamido]penicillanic acid trihydrate], penicillin G [sodium 6-(phenylacetamido)penicillanate], carbenicillin [disodium 6-(α-carboxyphenylacetamido)penicillanic acid], cloxacillin [sodium 6-[3-(2-chlorophenyl)-5-methylisoxazole-4-carboxamido]penicillanate monohydrate], floxacillin [sodium 6-[3-(2-chloro-6-fluorophenyl)-5-methylisoxazole-4-carboxamido]penicillanate monohydrate], methicillin [sodium 6-(2,6-dimethoxybenzamido)penicillanate monohydrate], phenethicillin [potassium 6-(α-phenoxypropionamido)penicillanate], penicillin V[1] [potassium 6-(phenoxyacetamido)penicillanate], propicillin [potassium 6-(α-phenoxybutyramido)penicillanate], and ticarcillin [disodium 6-[2-carboxy-2-(3-thienyl)acetamido]penicillanate]. All penicillins, except penicillin V (phenoxymethylpenicillin), were prepared in these laboratories.

Analytical Methods—Optical rotations were determined for the sodium D-line using a 10-cm cell[2]. Microanalyses were determined on a carbon–hydrogen–nitrogen analyzer[3], IR spectroscopy was carried out with potassium bromide disks[4], and NMR spectra[5] also were obtained.

TLC was performed on silica gel plates[6] with 1-butanol–ethanol–water (2:1:1) as the developing solvent. Detection was carried out with iodine vapor.

[1] Glaxo Limited.

[2] Perkin-Elmer model 141 polarimeter.
[3] Perkin-Elmer model 240.
[4] Perkin-Elmer model 457 spectrophotometer.
[5] Varian A60-A spectrometer.
[6] Eastman Kodak code 13181.

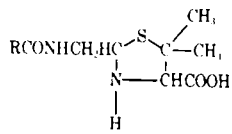


Table II—Penilloic Acids

Parent Penicillin	Formula		Analysis, % Calc.	Found	Equivalent Weight[a,b] Calc.	Found	$[\alpha]_D^b$	R_f (TLC)
Carbenicillin	$C_{16}H_{19}N_2O_5S \cdot \frac{1}{2}H_2O$	C	53.32	53.43	175.7	177.6	+31.8° (c 1.0, water + sodium hydroxide → pH 7.0)	0.54
		H	5.59	5.60				
		N	7.77	7.79				
		S	8.90	8.92				
Cloxacillin	$C_{18}H_{20}ClN_3O_4S$	C	52.72	52.32	409.9	401.4	+94.5° (c 1.0, water + sodium hydroxide → pH 7.0)	0.57
		H	4.92	4.88				
		Cl	8.76	8.58				
		N	10.26	10.18				
		S	7.83	7.77				
Floxacillin	$C_{18}H_{19}ClFN_3O_4S$	C	50.51	50.38	427.9	402.5	+44.6° (c 1.0, methanol)	0.66
		H	4.48	4.46				
		N	9.82	9.59				
		S	7.50	7.84				
Methicillin	$C_{16}H_{22}N_2O_5S$	C	54.22	53.95	354.4	332.0	+15.7° (c 1.0, methanol)	0.52
		H	6.26	6.45				
		N	7.90	7.66				
		S	9.05	8.93				
Penicillin G	$C_{15}H_{19}N_2O_3S \cdot H_2O$	C	55.20	55.38	326.4	319.4	+76.0° (c 1.0, methanol)	0.57
		H	6.79	7.04				
		N	8.58	8.56				
		S	9.82	9.92				
Penicillin V	$C_{15}H_{20}N_2O_4S$	C	55.53	55.42	324.4	323.2	+59.7° (c 1.0, methanol)	0.64
		H	6.21	6.27				
		N	8.66	8.47				
		S	9.88	10.26				
Ticarcillin	$C_{14}H_{18}N_2O_5S_2$	C	46.91	47.19	179.2	180.2	+49.0° (c 1.0, water + sodium hydroxide → pH 7.0)	0.45
		H	5.06	5.12				
		N	7.82	7.92				
		S	17.89	17.77				

[a] By aqueous titration. [b] Corrected for water.

Equivalent weights were determined by either of two methods. In the first method, carboxyl groups were determined by adding a known volume of standard sodium hydroxide to the test compounds, followed by back-titration with standard hydrochloric acid. In the second method, primary and secondary amino groups and metal cations were determined by titrating the test compounds under nonaqueous conditions with standard perchloric acid.

Iodometric assays for assessing the purity of penicilloic acids were carried out as described elsewhere (11), except that the initial alkaline hydrolysis stage was omitted. Values are quoted relative to analytical samples of the parent penicillins assayed by the standard iodometric method (11).

Preparation of Penicilloic Acids—Penicilloic acids were prepared by alkaline hydrolysis of the parent penicillins and isolated as described here. All products were dried *in vacuo* over phosphorus pentoxide. All preparations began with a stirred addition of the parent penicillin to water at ambient temperature. Sufficient 10 *M* NaOH was added to maintain the pH at 12.0 for 1.5 hr, *i.e.*, when the hydrolysis was complete. IR spectral analysis of the isolated compounds showed bands in the region of 1720 (carboxyl C=O), 1670 (amide C=O), and 1600 (aromatic C=C) cm^{-1}. No bands in the 1760–1785-cm^{-1} region (β-lactam C=O) were observed.

Amoxicillin Penicilloic Acid—Amoxicillin trihydrate (50 g) was hydrolyzed in water (500 ml). This solution was filtered, water was added to 1000 ml, and the pH was adjusted to 7.0 by addition of 5 *M* HCl. 2-Propanol (1000 ml) was added slowly with rapid stirring, and the solution was allowed to crystallize at 4°. The product was recovered as the monosodium salt by filtration, washed with cold 2-propanol–water (2:1), and dried, yielding 27 g (59%).

Ampicillin Penicilloic Acid—Ampicillin trihydrate (50 g) was hydrolyzed in water (250 ml). The solution was filtered, and the pH was adjusted to 7.0 by addition of 5 *M* HCl. 2-Propanol (700 ml) was added slowly with rapid stirring, and the resulting solution was allowed to crystallize at 4°. The product was recovered as the monosodium salt by filtration, washed with cold 2-propanol–water (3:1), and dried, yielding 24 g (53%).

Penicillin G Penicilloic Acid—Penicillin G sodium (100 g) was hydrolyzed in water (500 ml). The pH was adjusted to 5.4 by addition of concentrated hydrochloric acid. 2-Propanol (1500 ml) was added slowly with rapid stirring, and the resulting solution was allowed to crystallize at 4°. The product was recovered as the monosodium salt by filtration, washed with cold 2-propanol–water (3:1), and dried, yielding 58 g (55%).

Carbenicillin Penicilloic Acid—Carbenicillin disodium (30 g) was hydrolyzed in water (500 ml). The pH was adjusted to 1.8 by addition of 5 *M* HCl, and the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold water, and dried, yielding 9 g (31%).

Cloxacillin Penicilloic Acid—Cloxacillin sodium monohydrate (30 g) was hydrolyzed in water (150 ml). An equal volume of methyl acetate was added, and the pH was adjusted to 2.5 by addition of 5 *M* HCl with stirring. The organic phase was separated, and crystallization immediately occurred. The product was removed by filtration and dissolved in methanol (150 ml), and water (500 ml) was added slowly. The solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold water, and dried, yielding 14 g (45%).

Floxacillin Penicilloic Acid—Floxacillin sodium monohydrate (15 g) was hydrolyzed in water (250 ml). The pH was adjusted to 7.5 by addition of 5 *M* HCl, and the solution was concentrated by rotary evaporation to 70 ml. 2-Propanol (525 ml) was added slowly with stirring, and the solution was allowed to crystallize at 4°. The product was recovered as the disodium salt by filtration, washed with cold 2-propanol–water (7:1), and dried, yielding 9 g (57%).

Methicillin Penicilloic Acid—Methicillin sodium monohydrate (30 g) was hydrolyzed in water (150 ml). The pH was adjusted to 2.4 with 5 *M* HCl, and a white precipitate formed. The precipitate was then filtered and washed with water. Methyl acetate was added to the precipitate in sufficient quantity to dissolve the solid completely at 35–40°, and the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold methyl acetate, and dried.

Phenethicillin Penicilloic Acid—Phenethicillin potassium (22 g) was hydrolyzed in water (110 ml). The pH was adjusted to 7.0 by addition of 5 *M* HCl, and an equal volume of methyl acetate was added. The pH was then adjusted to 2.4 by slow addition of 5 *M* HCl with stirring, and the organic phase was separated and allowed to crystallize at 4°. The product

Table III—Inhibition of Hemagglutination by Penicilloic and Penilloic Acids

Parent Penicillin	Derived Penicilloic Acid: Molar Concentration Giving 50% Inhibition	Derived Penicilloic Acid: K_{rel}	Derived Penilloic Acid: Molar Concentration Giving 50% Inhibition	Derived Penilloic Acid: K_{rel}
Amoxicillin	7.81×10^{-4}	0.0078	—	—
Ampicillin	1.95×10^{-4}	0.0313	—	—
Carbenicillin	3.66×10^{-5}	0.1667	3.91×10^{-4}	0.1875
Cloxacillin	1.46×10^{-4}	0.0418	2.35×10^{-3}	0.0312
Floxacillin	3.91×10^{-4}	0.0156	1.56×10^{-3}	0.0470
Methicillin	3.91×10^{-4}	0.0156	2.35×10^{-3}	0.0312
Penicillin G	6.10×10^{-6}	1.0000	7.33×10^{-5}	1.0000
Penicillin V	1.22×10^{-5}	0.5000	2.93×10^{-4}	0.2502
Phenethicillin	3.66×10^{-5}	0.1667	5.86×10^{-4}	0.1251
Propicillin	7.33×10^{-5}	0.0832	7.81×10^{-4}	0.0939
Ticarcillin	7.81×10^{-4}	0.0078	2.35×10^{-3}	0.0312
Benzylpenicilloyl	5.72×10^{-7}	10.664	—	—

was recovered as the free acid by filtration, washed with cold methyl acetate, and dried, yielding 15.7 g (72%).

Penicillin V Penicilloic Acid—Penicillin V potassium (30 g) was hydrolyzed in water (150 ml). An equal volume of methyl acetate was added, and the pH was adjusted to 2.2 by addition of 5 *M* HCl with stirring. The organic phase was separated and allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold methyl acetate, and dried, yielding 17 g (57%).

Propicillin Penicilloic Acid—Propicillin potassium (22 g) was hydrolyzed in water (110 ml). An equal volume of methyl acetate was added, and the pH was adjusted to 2.4 by addition of 5 *M* HCl with stirring. The organic phase was separated and dried with magnesium sulfate. After removal of the drying agent, the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold methyl acetate, and dried, yielding 15 g (70%).

Ticarcillin Penicilloic Acid—Ticarcillin disodium (20 g) was hydrolyzed in water (100 ml). The pH was adjusted to 2.2 by addition of concentrated hydrochloric acid, and the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold water, and dried, yielding 4.0 g (21%).

Preparation of Penilloic Acids—Penilloic acids were prepared by acid decarboxylation of the parent penicilloic acids and isolated as described here. All were dried *in vacuo* over phosphorus pentoxide. IR spectral analysis of the isolated compounds showed bands in the regions of 1720 (carboxyl C=O), 1660 (amide C=O), and 1600 (aromatic C=C) cm^{-1}.

Penicillin G Penilloic Acid—Penicillin G sodium (100 g) was hydrolyzed in water (500 ml). The pH was adjusted to 4.0 by addition of 5 *M* HCl, and an equal volume of ethanol was added. The solution was heated at 70–80° until effervescence ceased while the pH was maintained at 4.0. The solution was filtered and then allowed to cool and crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold ethanol–water (1:1), and dried, yielding 49 g (57%).

Carbenicillin Penilloic Acid—Carbenicillin penicilloic acid (20 g) was added to water (500 ml), and the pH was adjusted to 1.8 by addition of 5 *M* HCl. On heating the suspension at 80°, the penicilloic acid dissolved and effervescence commenced with concomitant precipitation of the penilloic acid. When effervescence ceased, the suspension was cooled. The product was recovered by filtration, washed with cold water, and dried, yielding 11.3 g (65%).

Cloxacillin Penilloic Acid—Cloxacillin penicilloic acid (5 g) was added to a mixture of water (100 ml) and ethanol (100 ml), and the suspension was heated at ~80° until effervescence ceased. The solution was filtered and then allowed to cool and crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold ethanol–water (1:1), and dried, yielding 3.6 g (83%).

Floxacillin Penilloic Acid—Floxacillin sodium monohydrate (20 g) was hydrolyzed in water (100 ml). The solution was diluted to 350 ml (water), and the pH was adjusted to 3.0 by addition of 5 *M* HCl. The solution was heated at ~80° until effervescence ceased while the pH was maintained at <5.0. The pH was finally adjusted to 3.5, and the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with water, and dried, yielding 3.6 g (21%).

Methicillin Penilloic Acid—Methicillin sodium monohydrate (20 g) was hydrolyzed in water (100 ml). The pH was adjusted to 3.0 by addition of 5 *M* HCl, and the solution was heated at 80° while the pH was maintained at <5.0 by further additions of acid. When effervescence ceased, the pH was adjusted to 2.5, ethanol (60 ml) was added, and the solution was allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with water–ethanol (2:1), and dried, yielding 6.0 g (36%).

Penicillin V Penilloic Acid—Penicillin V penicilloic acid (5 g) was added to a mixture of ethanol (30 ml) and water (100 ml) and heated at 80° until effervescence ceased. The solution was cooled and allowed to crystallize at 4°. The product was recovered as the free acid by filtration, washed with cold water, and dried, yielding 3.1 g (74%).

Ticarcillin Penilloic Acid—Ticarcillin disodium (25 g) was hydrolyzed in water (250 ml). The pH was adjusted to 2.0 by addition of 5 *M* HCl, and the solution was heated at 80°. Effervescence began and the penilloic acid precipitated; when effervescence ceased, the solution was cooled. The product was recovered by filtration, washed with cold water, and dried, yielding 6.3 g (30%).

Amoxicillin and Ampicillin Penilloic Acids—All attempts to obtain pure samples of these compounds failed. On heating under acidic aqueous conditions, both ampicillin and amoxicillin penicilloic acids were decarboxylated as shown by the evolution of carbon dioxide. The reaction solutions turned yellow, and multiple products, as shown by TLC, were formed. One major product formed was fluorescent; with ampicillin, a compound with identical TLC characteristics was obtained by heating ampicillin itself under acidic conditions.

Phenethicillin and Propicillin Penilloic Acids—Pure crystalline samples of these materials could not be obtained. Both phenethicillin and propicillin penicilloic acids were decarboxylated on heating in acidic aqueous solution as shown by the evolution of carbon dioxide. The reaction products were extracted into methyl acetate and then into aqueous solution by addition of 2 *M* NaOH to pH 6.5. The aqueous phases were lyophilized to give products that were homogeneous by TLC (phenethicillin penilloic acid, R_f 0.64; propicillin penilloic acid, R_f 0.64) but which were not further characterized.

Selective Decarboxylation of Carbenicillin Penicilloic Acid—Carbenicillin disodium (20 g) was hydrolyzed in water (100 ml), and the solution was divided into two portions. To the first portion was added 5 *M* HCl to pH 2.0, and the solution was heated at 80°. The precipitate was removed when effervescence ceased, and TLC confirmed the precipitate to be carbenicillin penilloic acid.

The second portion of the original solution was heated at 100° at pH 12.0 for 1.5 hr, and the pH was maintained by further additions of alkali. TLC of the resulting solution indicated the presence of approximately equal amounts of carbenicillin penicilloic acid and a spot corresponding to penicillin G penicilloic acid. The solution was adjusted to pH 2.0 by addition of 5 *M* HCl and heated at 80° until effervescence ceased. The precipitate was removed and examined, along with the supernate, by TLC. The precipitate contained one major component corresponding to carbenicillin penilloic acid and a trace component corresponding to penicillin G penilloic acid. The supernate contained one major component corresponding to penicillin G penilloic acid and a trace component corresponding to carbenicillin penilloic acid.

***N*-Benzylpenicilloyl ε-Aminocaproic Acid**—The preparation of this compound was described elsewhere (12).

Preparation of Benzylpenicilloyl Antiserums—Benzylpenicilloylated bovine γ-globulin was prepared as described previously (13). New Zealand White rabbits, 2.5 kg, were immunized by 0.5-ml subcutaneous and intramuscular injections of immunogen at a concentration of 10 mg/ml, emulsified in Freund's complete adjuvant, along with 5 × 0.1-ml intradermal injections of immunogen at 10 mg/ml in 0.15 *M* saline. At 2, 4, 5, and 6 months, the animals were each given a booster subcutaneous injection of 2 mg of immunogen in 0.15 *M* saline. Three weeks after the final booster injection, the animals were exsanguinated.

Sensitization of Rabbit Erythrocytes and Standardization of Antiserums for Hemagglutination Studies—The method used to sensitize the rabbit erythrocytes was similar to that described by Thiel *et al.* (14).

Blood from a donor rabbit was mixed with an equal volume of Alsever's solution. The cells were washed three times in pH 7.2 phosphate-buffered saline and made up to 20% in 0.14 *M* barbital[7] buffer, pH 8.5. To 30 ml of the cell suspension was added 1.0 g of penicillin G potassium (benzylpenicillin potassium), followed by incubation at 37° for 1 hr. The cells were washed six times in phosphate-buffered saline containing 1% normal rabbit serum and resuspended to a final concentration of 2% (v/v) in this medium.

[7] Veronal.

Table IV—NMR Spectra for Penilloic Acids

Parent Penicillin	Solvent	Ar	ArC*H*	CH_2CHS	CH_2CHS	OCH_2CO	$(CH_3)_2CCH$	$(CH_3)_2C$	CCH_3
Carbenicillin	Pyridine-d_5 plus deuterium oxide	7.35 (m, 5)	Plus CH_2CHS 5.16 (m, 2)	Plus $(CH_3)_2CCH$ 3.95 (m, 3)	—	—	—	1.70 (d, 6)	–
Cloxacillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	7.54 (s, 4)	—	4.71 (t, 1)	3.26 (d, 2)	—	3.56 (s, 1)	1.39 (d, 6)	2.65 (s, 3)
Floxacillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	7.34 (m, 3)	—	4.72 (t, 1)	Plus $(CH_3)_2CCH$ 3.0–3.7 (m, 3)	—	—	1.40 (d, 6)	2.69 (s, 3)
Methicillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	6.5–7.6 (m, 3)	—	4.77 (t, 1)	Plus $(OCH_3)_2$ plus $(CH_3)_2CCH$ 3.4–4.9	—	—	1.43 (d, 6)	–
Penicillin G	Acetone-d_6 plus deuterium oxide	7.36 (s, 5)	3.61 (s, 2)	4.87 (t, 1)	3.41 (d, 2)	—	3.78 (s, 1)	1.47 (d, 6)	–
Penicillin V	Dimethyl sulfoxide-d_6 plus deuterium oxide	6.8–7.6 (m, 5)	—	4.80 (t, 1)	—	4.53 (s, 2)	Plus CH_2CHS 3.2–3.8	1.42 (d, 6)	–

Cells for control experiments were treated in an identical fashion, except that the penicillin G was omitted.

Serial twofold dilutions of the rabbit antibenzylpenicilloyl antiserums and serums from control nonimmunized rabbits were prepared. To 0.05-ml portions of the appropriate antiserum dilutions were added 0.05 ml of phosphate-buffered saline and 0.05 ml of penicilloylated, or control, erythrocytes. The mixtures were incubated at 37° for 1 hr, and the results were read. From these experiments, an antiserum was selected and used for the inhibition experiments described here at a dilution of 1:50, the highest dilution giving complete agglutination of the benzylpenicilloylated red cell preparation.

Hemagglutination Inhibition by Penicilloic and Penilloic Acids—Samples of the test penicilloic and penilloic acids were prepared as 0.0125 *M* solutions in phosphate-buffered saline containing 1% normal rabbit serum, and serial twofold dilutions of each solution were made. To 0.05-ml portions of each inhibitor dilution was added 0.05 ml of rabbit

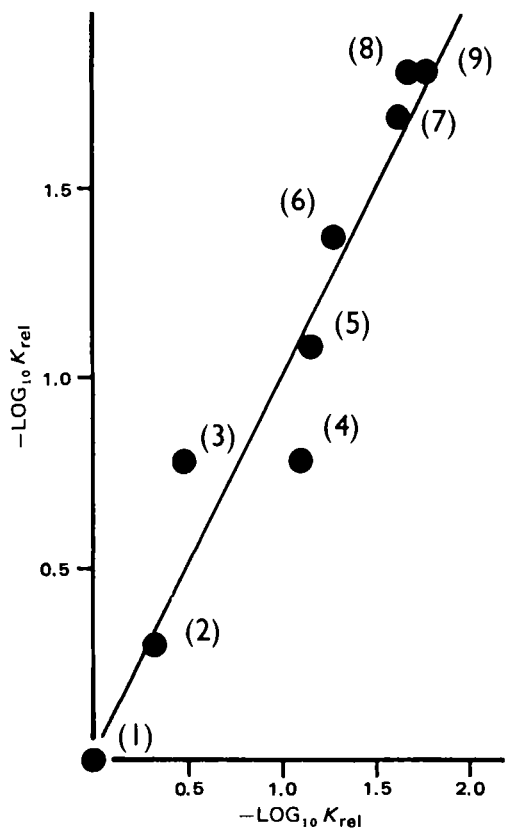


Figure 1—*Correlation (r = 0.967, p < 0.001) between hemagglutination inhibition by penicilloic acids and precipitation inhibition by alkaline hydrolysis products of corresponding penicillins. The $-\log_{10} K_{rel}$ data obtained in this study were plotted* versus *the $-\log_{10} K_{rel}$ data from Nishida* et al. *(8). Key: (1), penicillin G; (2), penicillin V; (3), carbenicillin; (4), phenethicillin; (5), propicillin; (6), cloxacillin; (7), ampicillin; (8), floxacillin; and (9), methicillin.*

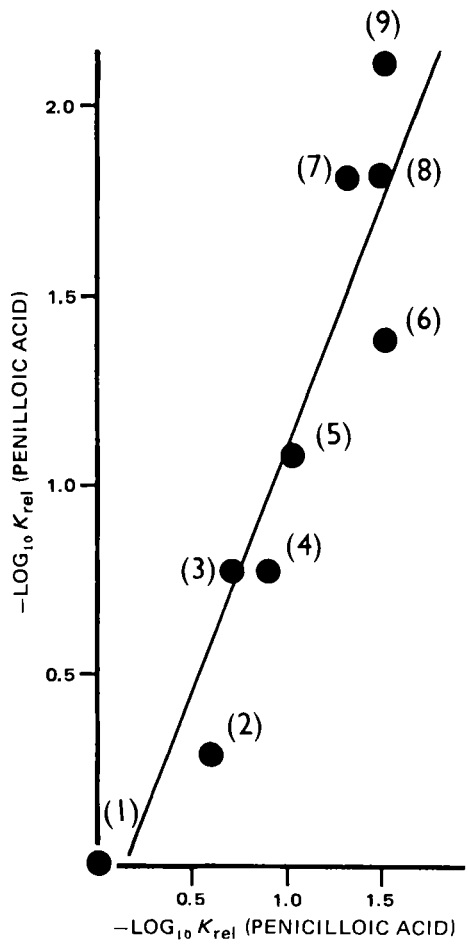


Figure 2—*Correlation (r = 0.937, p < 0.01) between hemagglutination inhibition by penicilloic acids and corresponding penilloic acids. Key to parent penicillins: (1), penicillin G; (2), penicillin V; (3), carbenicillin; (4), phenethicillin; (5), propicillin; (6), cloxacillin; (7), floxacillin; (8), methicillin; and (9), ticarcillin.*

Table V—NMR Spectra for Penicilloic Acids

Parent Penicillin	Solvent	Ar	Ar*CH*	ArCH_2	CHC*HS*	*CH*CHS	*CHCHS*	$(OCH_3)_2$	OCH_2-CO	$(CH_3)_2$-CC*H*	$(CH_3)_2C$	CCH_3
Amoxicillin	Deuterium oxide plus sodium deuteroxide	6.85 (q, 4)	4.45 (s, 1)	—	5.06 (d, 1)	4.22 (d, 1)	—	—	—	3.34 (s, 1)	1.29 (d, 6)	—
Ampicillin	Deuterium oxide	7.55 (s, 5)	5.20 (s, 1)	—	5.07 (d, 1)	4.28 (d, 1)	—	—	—	3.06 (s, 1)	1.05 (d, 6)	—
Carbenicillin	Methanol-d_4	7.45 (s, 5)	4.80 (s, 1)	—	—	—	5.20 (m, 2)	—	—	3.67 (d, 1)	1.36 (q, 6)	—
Cloxacillin	Methanol-d_4	7.53 (s, 4)	—	—	5.03 (d, 1)	4.56 (d, 1)	—	—	—	3.60 (s, 1)	1.43 (d, 6)	2.73 (s, 3)
Floxacillin	Deuterium oxide	7.48 (m, 3)	—	—	5.06 (d, 1)	4.35 (d, 1)	—	—	—	3.46 (s, 1)	1.43 (d, 6)	2.78 (s, 3)
Methicillin	Deuterium oxide plus sodium bicarbonate	6.6–7.7 (m, 3)	—	—	5.59 (d, 1)	4.92 (d, 1)	—	3.89 (s, 6)	—	4.21 (s, 1)	1.61 (d, 6)	—
Penicillin G	Acetone-d_6 plus deuterium oxide	7.36 (s, 5)	—	Plus $(CH_3)_2CCH$ 3.70 (s, 3)	5.18 (d, 1)	4.57 (d, 1)	—	—	—	—	1.40 (d, 6)	—
Penicillin V	Dimethyl sulfoxide-d_6 plus deuterium oxide	6.8–7.6 (m, 5)	—	—	5.10 (d, 1)	4.49 (d, 1)	—	—	4.62 (s, 2)	3.66 (s, 1)	1.40 (d, 6)	—
Phenethicillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	6.8–7.6 (m, 5)	—	—	5.15 (d, 1)	Plus OC*H* 4.4–5.0 (m, 2)	—	—	—	3.71 (s, 1)	Plus $OCHCH_3$ 1.1–1.9 (m, 9)	—
Propicillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	6.8–7.6 (m, 5)	—	—	5.11 (d, 1)	Plus $OCHCH_2CH_3$ 4.3–4.9 (m, 2)	—	—	—	3.69 (s, 1)	Plus $OCHCH_2CH_3$ 0.7–2.2 (m, 11)	—
Ticarcillin	Dimethyl sulfoxide-d_6 plus deuterium oxide	7.34 (m, 3)	Plus *CHCHS* 5.00 (m, 3)	—	—	—	—	—	—	3.54 (d, 1)	1.3 (q, 6)	—

benzylpenicilloyl antiserum at a dilution of 1:50; the mixtures were then incubated at 37° for 1 hr. To each dilution was added 0.05 ml of penicilloylated erythrocytes, and the mixtures were incubated for a further 1 hr at 37°; then the results were read.

All inhibition experiments were carried out simultaneously with the same batch of penicilloylated erythrocytes.

Calculations—For each inhibitor, the dilution and the corresponding concentration giving 50% inhibition of hemagglutination were determined. Values for the relative association constants, K_{rel}, for each inhibitor were calculated as previously described (8), where:

$$K_{rel} = \frac{[\text{reference hapten}]_{50\%}}{[\text{test hapten}]_{50\%}} \qquad \text{(Eq. 1)}$$

In the series of penicilloic acids, the reference hapten chosen was benzylpenicilloic acid; in the series of penilloic acids, benzylpenilloic acid was selected.

RESULTS AND DISCUSSION

Preparation of Penicilloic and Penilloic Acids—The penicilloic acids of a number of penicillins were isolated in pure crystalline form, and the analytical data obtained for these materials are given in Table I. Whereas most compounds were isolated as the free acids, some, notably the penicilloic acids of the amino penicillins amoxicillin and ampicillin, were more readily isolable as the sodium salts.

Under the experimental conditions, it is unlikely that polymers, known to form in concentrated solutions of ampicillin kept at a high pH (15), would have been present in the final preparation. The hydrolysis time was quite short, and workup involved crystallization and washing.

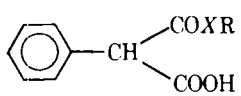
All penicilloic acids examined readily decarboxylated on heating in acidic solution, and several pure penilloic acids were obtained in this fashion. The analytical data obtained for these materials are given in Table II. Whereas most penicilloic acids yielded one main product by this treatment, amoxicillin and ampicillin penicilloic acids yielded multiple products, as shown by TLC analysis. One of these products was of a fluorescent yellow nature, and a fluorescent compound with identical TLC characteristics could be identified in solutions of ampicillin heated under acidic conditions at concentrations of 1 mg/ml.

A second fluorescent component also was formed under these conditions; at ampicillin concentrations of 50 μg/ml, only this second component was formed. The formation of this second component was reported by Jusko (16), who suggested that in solutions of ampicillin penicilloic acid at low pH the 7-carboxyl group could condense with the α-amino group to form a substituted 3,6-diketopiperazine.

The penilloic acids of phenethicillin and propicillin could not be obtained in crystalline form. The products of the decarboxylation of the respective penicilloic acids were purified by extraction procedures and shown to be homogeneous by TLC, with migration characteristics typical of the other penilloic acids. Although these materials were not analyzed further, they were tested by hemagglutination inhibition for comparative purposes.

The decarboxylation of carbenicillin penicilloic acid yielded interesting results; selective removal of the 7-carboxyl group or the side-chain α-carboxyl group could be achieved, depending on the experimental conditions chosen. At low pH values, the 7-carboxyl group was removed to give carbenicillin penilloic acid. This reaction is typical of penicilloic acids, although the mechanism of the decarboxylation involved has not been elucidated (17). However, at higher pH values, the side-chain carboxyl group was selectively removed to yield penicillin G penicilloic acid. This type of reaction appears to occur with carbenicillin itself since penicillin G may be found as an impurity in preparations of this penicillin (18). In terms of the mechanism of removal of the α-carboxyl group, the side chain in carbenicillin corresponds to a substituted phenylmalonic acid (IV), and it has been found[8] that the carboxyl groups in IV and V are susceptible to decarboxylation in the presence of base.

Immunological Cross-Reactivity of Penicilloic and Penilloic Acids—The penicilloic acids isolated were examined for their ability to interact with antibenzylpenicilloyl antibodies by hemagglutination inhibition measurements, and the results are shown in Table III. The interaction between antibenzylpenicilloyl antibodies and the unpurified alkaline hydrolysis products of various penicillins was studied by Nishida *et al.* (8). The results of their studies are in good agreement with those detailed here, and Fig. 1 depicts a significant correlation between both sets of data.



IV: X = NH
V: X = O

The penilloic acids isolated also inhibited antibenzylpenicilloyl antibodies (Table IV); on average, an 11-fold increase in the concentration of a penilloic acid was required to produce the same degree of inhibition as was given by the corresponding penicilloic acid. However, there was a significant correlation between the inhibitory capacities of penicilloic acids and the corresponding penilloic acids (Fig. 2). This result is not unexpected since it is known that the side chain in penicillins (and in cephalosporins) plays an important part in the immunological recognition of the penicilloyl group (19, 20).

A Hansch analysis of the inhibition data obtained in this study was reported recently (21), illustrating the importance of hydrophobic and steric effects on the inhibitory activities of the haptens. The fact that even slight modifications of the side chain of benzylpenicilloic acid, *e.g.*, substitution of α-H by α-NH_2, can markedly affect the reactivity of this hapten toward benzylpenicilloyl antibodies is paralleled by the reported results of skin tests with penicillin-allergic individuals. Parker and Thiel (22) skin tested patients with the penicilloyl polylysine derivatives of penicillin G, phenethicillin, and methicillin, and different patterns of reactivity among patients were observed with the reagents.

Van Dellen *et al.* (6) conducted similar tests with the penicilloyl polylysine derivatives of penicillin G, methicillin, and ampicillin and again a varied pattern of reactivity was found; some patients responded to one reagent, some to two, and only a few to all three. The penicillin G derivatives most frequently gave reactions, resulting in positive skin tests in 90% of the patients; at the other extreme, the ampicillin derivative gave positive skin tests in 22% of the patients.

The same varied pattern has been observed in *in vitro* tests for penicilloyl antibodies from allergic patients. Using the radioallergosorbent test (RAST) technique to measure specific IgE antibodies, Juhlin and Wide (23) found the serum of some penicillin-allergic patients to contain equivalent antibody titers to the penicilloyl groups derived from penicillin G and penicillin V. On the other hand, some patient serums gave considerably higher titers with one or another of the penicillins.

In terms of clinical allergy, it would be tempting to speculate that appropriate side-chain modifications within penicillins and cephalosporins could reduce the risk of hypersensitivity reactions. In this context, cefazolin, a cephalosporin antibiotic containing a tetrazolylacetyl side chain, has been shown (20, 24) to have a low degree of cross-reactivity with penicillin G and cephaloridine in experimental animal models. However, most studies on the cross-reactions between various penicillins and between penicillins and cephalosporins have been related to the cross-reaction with antibenzylpenicilloyl antibodies. The present study is no exception; but to understand the complex events of penicillin allergy in more detail, studies must be extended to those antibodies derived from penicilloyl groups other than penicillin G. The preparation of pure haptens, as described here, may enable such studies to be carried out in a more facile manner.

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Dequaternization of Curare Bases with Sodium Thiophenoxide and Ethanolamine

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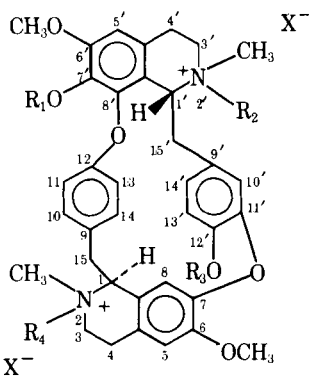
Abstract □ To prepare (+)-tubocurine and *O,O*-dimethyl-(+)-tubocurine, the commonly used dequaternization procedures with sodium thiophenoxide and ethanolamine were investigated. The quaternary compounds were (+)-tubocurarine chloride and the chloride and iodide salts of *O,O*-dimethyl-(+)-chondocurarine. The results obtained with ethanolamine indicate that Hofmann elimination is a major pathway and that *N*-demethylation is minor. The elimination products of *O,O*-dimethyl-(+)-chondocurarine iodide with ethanolamine were identified as *O,O*-dimethyltubocurinemethine, *O,O*-dimethyltubocurineisomethine, and *O,O*-dimethyltubocurinedimethine. *N*-Demethylation was the primary reaction with sodium thiophenoxide. Thus, dequaternization of (+)-tubocurarine chloride with sodium thiophenoxide provided (+)-tubocurine which, on diazomethylation, yielded *O,O*-dimethyl-(+)-tubocurine, identical to the compound obtained by *N*-demethylation of *O,O*-dimethyl-(+)-chondocurarine chloride with the same reagent.

Keyphrases □ (+)-Tubocurine—syntheses by dequaternization procedures compared, mechanisms evaluated, products identified □ *O,O*-Dimethyl-(+)-tubocurine—syntheses by dequaternization procedures compared, mechanisms evaluated, products identified □ Dequaternization—of various curare bases, different procedures compared, mechanisms evaluated, products identified □ Curare bases, various—syntheses by dequaternization procedures compared, mechanisms evaluated, products identified

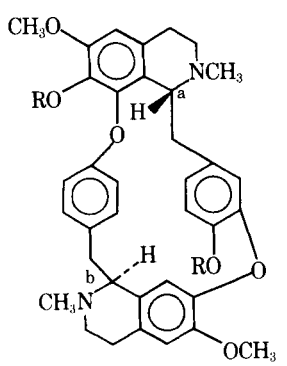
The accepted structure of (+)-tubocurarine chloride (1) was revised to Structure I previously (2). These investigators indicated that (+)-tubocurarine chloride is not a diquaternary compound and that (+)-tubocurine (II) and (+)-chondocurine have identical structures (II) and do not differ as previously reported (3–6). The revelation that (+)-tubocurarine was actually a monoquaternary–monotertiary species suggested that selective monoquaternization of the ditertiary amine, (+)-tubocurine, could provide a pair of isomeric monoquaternary–monotertiary tubocurarines, namely, semisynthetic (+)-tubocurarine and (+)-isotubocurarine chloride. The preparation of the latter was described previously (7). Although (+)-chondocurine [(+)-tubocurine] (II) has been isolated from natural sources (5, 6), the fact that these sources are somewhat inaccessible suggested the generation of II from commercially available I by dequaternization.

Dealkylation of quaternary ammonium salts is a problem that has received considerable attention, but most of the various methods employed (8–16) have limitations. For example, Tomita and Takano (17) showed that the widely used method of heating the quaternary salt in refluxing ethanolamine may lead to extensive Hofmann elimination as well as to *O*-demethylation.

To prepare II, Shamma *et al.* (18) utilized sodium



I: $R_1 = R_3 = R_4 = H$, $R_2 = CH_3$, X = Cl
III: $R_1 = R_2 = R_3 = R_4 = CH_3$, X = I
IV: $R_1 = R_2 = R_3 = R_4 = CH_3$, X = Cl



II: R = H, a,b = *R,S*
V: R = CH_3, a,b = *R,S*

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