THE PREPARATION AND DETERMINATION OF STRUCTURE OF BENZYLPENICILLOYL COMPOUNDS USED IN SKIN-TESTING FOR PENICILLIN ALLERGY

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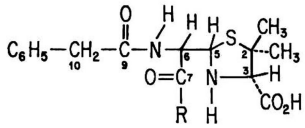
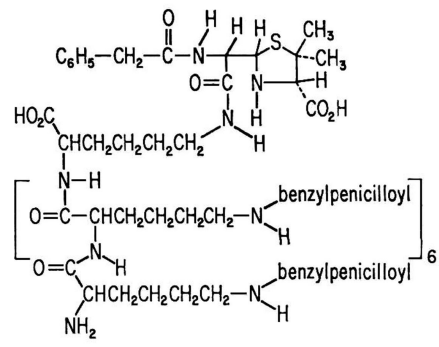
The preparation of α-D-benzylpenicilloyl-*n*-propylamine and octa-ε-(α-D-benzylpenicilloyl)-octa-α-L-lysine are described. Their structures were established by chemical and spectroscopic evidence. Proton and carbon-13 nuclear magnetic resonance spectra of these two penicillin derivatives and some related compounds are provided. These compounds are useful in skin testing for penicillin allergy.

There is considerable theoretical and clinical interest in a reliable predictive test for penicillin allergy. Previous studies from this and other laboratories demonstrate that intradermal skin testing with appropriate reagents constituted such a test[1~5]. The reagents we used previously for testing were 1) penicilloyl-polylysine and 2) a minor determinant mixture consisting of penicillin, its hydrolysis product, penicilloic acid, penicilloyl-amide and penilloic acid[1]. More recently an improved set of skin testing materials has been devised, and is currently being used in a multi-center clinical trial sponsored by the NIAID (National Institute of Allergy and Infectious Diseases, USA). The new reagents are: 1) octa-ε-(α-D-benzylpenicilloyl)-octa-α-L-lysine, 2) a minor determinant mixture consisting of benzylpenicillin, benzylpenicilloic acid and benzylpenicilloyl-*n*-propylamine.

Two of these substances, D-benzylpenicillin and D-benzylpenicilloic acid (**I**) are well known and their structures established. The preparation, properties and assignment of structure of the other substances, α-D-benzylpenicilloyl-*n*-propylamine (**II**) and octa-ε-(α-D-benzylpenicilloyl)-octa-α-L-lysine (**III**), have not been previously reported. The purpose of this communication is to describe the preparation of these materials, and provide chemical and spectroscopic evidence that substantiates their assigned structures.



I R=OH
II R=NHCH$_2$CH$_2$CH$_3$



III

Results and Discussion

Some preliminary evidence for the assignment of structure of compounds **II** and **III** comes from the method of synthesis and infrared spectroscopy. The most electrophilic center of an intact penicillin is

the carbonyl group of the β-lactam; thus reaction of benzylpenicillin with nucleophiles such as the amino group of *n*-propylamine or the ε-amino group of lysine would be expected to cleave the β-lactam and yield a penicilloyl compound. The absence of infrared absorption in the 1780~1770 cm^{-1} region, characteristic of fused β-lactam carbonyl groups coupled to thiazolidine rings[6], in compounds **II** and **III** is indicative that they are penicilloyl compounds.

Data on the proton magnetic resonance spectra of benzylpenicilloic acid (**I**) along with other penicilloic acids and penilloic acids were reported recently[7], but not analyzed. Our data (see Table 1) are in general agreement with the reported data. An analysis of our proton magnetic resonance data aids in substantiating the structure assigned compound **II**. The close similarity of the chemical shifts for the H-3, CH_2 and aromatic protons of the two penicilloyl compounds (**I** and **II**) contrast to the chemical shifts noted for the same protons in the parent penicillin. More notable are the chemical shift differences ($\Delta\delta$) of the C-2 *gem* dimethyl protons. In benzylpenicillin, with an intact β-lactam, the $\Delta\delta$ is 17 ppm, while in compounds **I** and **II** (lacking the β-lactam) the $\Delta\delta$ is 25 and 33 ppm, respectively. In penicillin the δ of the H-5 and H-6 protons is nearly the same, and when DMSO-d_6 is the solvent, the resonance peaks appear as a complex multiplet. In compounds **I** and **II** the H-5 and H-6 resonance absorption move to a lower field with a greater chemical shift between them, and in the absence of deuterium oxide the H-5 protons are doublets (J=5~7 Hz) and the H-6 protons are triplets (J=7~8 Hz).

The carbon-13 magnetic resonance spectra of a number of penicillins have been reported[8~11]. These are quite useful for assigning structures and conformations, along with potentially predicting the chemical and biological activity [8].

We found that the ^{13}C magnetic resonance chemical shifts of octa-α-L-lysine closely resemble those reported for L-lysine[12]. These are both shown in Table 2. The assignments of the ^{13}C chemical shifts for benzylpenicilloic acid (**I**) ,benzylpenicilloyl-*n*-propylamine (**II**) and octa-ε-benzylpenicilloyl-octa-α-L-lysine (**III**) are based on the reported lysine[12] and penicillin spectra[8,11]. Again, as in the ^1H NMR spectra, the ^{13}C chemical shifts of the penicilloyl compounds **II** and **III** are more alike one another than are the shifts of benzylpenicillin. Interestingly, the C-2 *gem* dimethyl carbons chemical shift changes from the intact β-lactam structure (benzylpenicillin) *vs* the penicilloyl structures, but here the $\Delta\delta$ decreases on ring cleavage.

The elemental analysis, method of preparation, *etc.* along with the ^1H and ^{13}C NMR spectra described above substantiate the structure of compound **II**. The same can be said for compound **III**, but as it is a more complex molecule, some additional data were obtained to verify its structure. As octa-α-L-lysine was prepared on a Merrifield resin one is concerned that the isolated product was solely an octapeptide. Upon thin-layer chromatography the purported octa-α-L-lysine could be distinguished from samples of known di, tri, tetra and penta-α-L-lysines[13]. Within the limits of the assay, the sample consisted of a homogeneous polylysine, and according to its relative Rf a polylysine, larger than penta-lysine. In the preparation of compound **III** the octa-α-L-lysine was allowed to react with an excess of

Table 1. Proton chemical shift assignments (δ, ppm).

Compound	$(CH_3)_2$	H-3	H-5, H-6	CH_2	Aromatic H	Other
Benzylpenicillin	1.47, 1.62	3.87	5.37 (m)	3.55	7.23	
Benzylpenicilloic acid (**I**)	1.22, 1.47	3.64	5.10 (d, J=5 Hz)	3.60	7.30	
Benzylpenicilloyl-*n*-propyl-amine (**II**)	1.22, 1.55	3.68	4.92 (d, J=7 Hz) 4.43 (t, J=8 Hz)	3.62	7.33	*N*-propyl (α) 3.02, (β) 1.35, (γ) 0.83

Table 2. ^{13}C Chemical shift assignments (δ, ppm).

Compound	Cα	Cβ	Cγ	Cδ	Cε	C-2	C-3	C-5	C-6	C-10	αCH$_3$	βCH$_3$	CO	Aromatic
L-Lysine[a]	55.3	27.2	22.4	30.7	40.0								175.4	
Octa-α-lysine	56.5 58.2	29.1	22.8	33.4	42.0								176.3 188.0	
Benzylpenicillin[b]						65.2	73.9	67.4	58.9	48.2	27.9[c]	31.0[c]	174.1 174.7 175.3	
Benzylpenicilloic acid (I)						62.7	78.2	68.9	61.3	45.2	29.0	29.5	176.9 178.2 178.8	130.1 131.8 132.2
Benzylpenicilloyl-*n*-propyl amine[d] (II)	44.9	24.5	13.4			62.1	77.3	67.5	61.9	43.9	28.5	29.8	173.9 177.2 177.7	130.1 131.7 132.0
Octa-ε-benzylpenicilloyl-octa-α-L-lysine (III)	56.8	29.6	25.2	33.6	41.9	61.9	78.6	69.1	60.6	45.1	30.5	31.0	[e]	130.7 131.8 137.5

a) These assignments are found in reference 12.
b) The C-2 ~ C-10 and CO assignments are found in reference 11.
c) The αCH$_3$ and βCH$_3$ assignments are from phenoxymethyl penicillin found in reference 8.
d) The Cα, Cβ, Cγ assignments are for the *n*-propylamide.
e) A minimum of six weak peaks in the 178 ~ 188 ppm region were observed.

benzylpenicillin. Previous investigations in our laboratory showed that if a comparable sized polylysine was not completely converted to its penicilloyl derivative (*i.e.* by allowing fewer equivalents of penicillin to react with the polylysine) the products formed failed to precipitate at pH 3.6. When compound **III** was isolated it precipitated at pH 3.6 and was then resolubilized and then reprecipitated. The elemental analyses (C, H, N and S) of compound **III** are consistent with the assigned structure. In addition, the amount of amino nitrogen was found to be one half the Kjeldahl total nitrogen. Penamaldate analysis for penicilloyl groups, which was developed by one of us[14], of compound **III** showed eight penicilloyl equivalents per mole of compound **III**.

Experimental

Preparation of Benzylpenicilloic Acid (I)

This is a known compound whose preparation has been described in detail[15].

Preparation of Benzylpenicilloyl-*n*-propylamine (II)

Twenty six gram (0.07 mole) of potassium benzylpenicillin was dissolved in 20 ml of water. Freshly distilled *n*-propylamine 6.2 g (0.10 mole) was added dropwise to the stirred penicillin solution at room temperature under pH control. A TTT No. 1 Radiometer was used to monitor and adjust the pH in this experiment and in the other experiments described below. The pH was maintained at 11.2~11.5 for the first 5 minutes, during which time about 0.07 moles of *n*-propylamine was added. After that, the pH rose to 11.8~12.0 as the additional *n*-propylamine was added in the next 5 minutes. The mixture was then stirred at room temperature for an additional 60 minutes. The resulting solution was cooled to 5~10°C in an ice bath, and the pH adjusted to 3.8~4.0 by additions of 3 N HCl to the stirred reaction mixture. The resulting white precipitate was separated by filtration and washed extensively with cold water. The moist solid was dried under high vacuum. Twenty grams of a white powder (70%) was obtained which melted at 107~109°C.

Anal. Calcd. for $C_{19}H_{27}N_3O_4S \cdot \frac{1}{2}H_2O$: C 56.70, H 7.01, N 10.44, S 7.97.
Found: C 56.88, H 6.84, N 10.31, S 8.13.

Preparation of Octa-α-L-lysine Nanohydrobromide

This material was prepared by Vega-Fox Laboratory, Tuscon, Arizona. The starting material was α-amino-*tert*-butyloxycarbonyl-ε-aminobenzyloxycarbonyl-α-carboxy Merrifield resin ester of L-lysine. Using trifluoroacetic acid to unblock the α-amino group, seven consecutive couplings with α-amino-*tert*-butyloxycarbonyl-ε-aminobenzyloxycarbonyl lysine as the addend and using dicyclohexylcarbodiimide as the coupling reagent were carried out. The resulting octa-α-L-lysine was deprotected and cleaved from the resin by mixing the material in trifluoroacetic acid saturated with gaseous hydrogen bromide (1 g of solid per ml) for 25 minutes at room temperature. The excess hydrogen bromide was removed by purging with nitrogen and the trifluoroacetic acid removed by evaporation under vacuum. The residue was dissolved in water and lyophilized. This material was then dissolved in 50% acetic acid and chromatographed on a Sephadex G-15 column. The solution containing the major peak was diluted with water to a 10% acetic acid solution and then lyophilized to yield a slightly yellow amorphous powder[16].

Thin-layer chromatography of this powder in our laboratory on MN 300 cellulose plates with isopropanol, acetic acid, water and pyridine (30: 6: 24: 70, v/v) as the eluent, and using a ninhydrin spray as the detector showed a single spot, Rf 0.08. Samples of di, tri, tetra and penta-α-L-lysine when chromatographed in the same system had Rf(s) distinguishable from that of the octa-α-L-lysine. This analytical method is based on the prior paper chromatographic method developed by LEVINE and REDMOND[13].

Preparation of Octa-ε-(α-D-benzylpenicilloyl)-octa-α-L-lysine (III)

One hundred mg octa-α-L-lysine nonahydrobromide (assuming 90% peptide, 10% water, 0.38 meq) was dissolved in 25 ml deionized water and the pH was adjusted to 10±0.3 with 0.5 M sodium hydroxide.

A solution of 755 mg of potassium benzylpenicillin (2.0 meq) in 10 ml of water was added and the pH was brought to 11.5±0.05. The reaction proceeded at room temperature stirred by a magnetic stirrer and the pH was maintained at 11.5±0.05 by additions of the 0.5 M sodium hydroxide. After two hours, when the reaction had gone to completion as evidenced by stability of the pH at 11.5, the solution was cooled to 5~10°C. The pH was adjusted to 3.6 by additions of 1 N hydrochloric acid to the stirred solution and a white precipitate formed. The cold solution was then centrifuged, the supernatant removed and the precipitate washed twice with small quantities of cold deionized water. The precipitate was then suspended in 20 ml of deionized water and dissolved by adjusting the pH to 9.5~10.5 with sodium hydroxide. The solution was clarified by cold centrifugation and while cold the pH adjusted to 3.6 with 1 N hydrochloric acid. The white precipitate which formed was centrifuged and washed three times with small quantities of ice-cold deionized water. The precipitate was dried under high vacuum and a yield of 104 mg of a white powder (55%) was obtained.

Anal. Calcd. for $C_{176}H_{242}N_{32}O_{41}S_8 \cdot 16H_2O$: C 54.63, H 6.89, N 11.58, S 6.63.
Found: C 54.45, H 6.59, N 11.61, S 6.57.

Penamaldate Analysis

The laboratory procedures described by LEVINE[14] were used. The average optical density of two samples (≃1 mg/ml) of octa-ε-benzylpenicilloyl-octa-α-L-lysine[3] at 285 nm in 0.05 M sodium phosphate, after correcting for reagent absorption and dilution error, were found to be 45.8. The molar extinction coefficient of the benzylpenicilloyl moiety in the penamaldate assay has been shown to be 23,500[14]. Thus the benzylpenicilloyl concentration was 1.90×10^{-3} M. The total nitrogen content of this sample was determined by the micro-Kjeldahl method and was found to be 106 μg/ml. Thus the nitrogen from the benzylpenicilloyl moiety was equal to 1.90×10^{-3} mmole/ml × 28 μg nitrogen/mmole benzylpenicilloyl or 53 μg/ml. This is exactly what would be predicted if the number of lysine moieties equals the number of benzylpenicillin moieties as they each contain two nitrogen atoms/moiety. The total polylysine-8 concentration was then calculated based on 53 μg nitrogen/ml × 130.2 mg/mole of lysine in octalysine monohydrate × 1 mole lysine/28 mg nitrogen × 1 mole lysine-8/1,042 mg and found to be 2.37×10^{-4} M. Thus the number of benzylpenicilloyl groups per mole polylysine-8 was found to be 1.90×10^{-3} M/2.37×10^{-4} M or 8.02.

NMR Spectra

^{1}H NMR spectra were determined on a 60 megacycle Varian A60A spectrometer. Samples were in dimethyl sulfoxide-d_6 and tetramethylsilane was the internal standard. The ^{13}C NMR spectra were determined at 75 megahertz on a Bruker WM-300 spectrometer using pulse Fourier transform techniques with proton decoupling. Samples were in deuterium oxide in 10 mm tubes. The octalysine octahydrobromide was dissolved in the solvent, while the pH of the other samples was adjusted to 9.3~9.4 with sodium deuteroxide. The internal standard was *p*-dioxane and the chemical shifts were converted to tetramethylsilane by using the known difference of 67.4 ppm.

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