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Antibiotic Polymers: α-Aminobenzylpenicillin (Ampicillin)†

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Abstract—Ampicillin (α-aminobenzylpenicillin) partially degrades and polymerizes spontaneously in aqueous solution to yield ordered macromolecules with molecular weight varying between 1000 and over 5000. As studied by spectrometry (UV, IR and NMR), chemical and microbiological reactivity, the polymers are formed at least in part by subunits of intact ampicillin and its penicilloic acid derivative, the proportion of the latter increasing with molecular weight. The resulting dimers, trimers and polymers vary in configuration according to which of several functional groups establish covalent bonds with each other but show in general a partial loss of cyclic structure with preservation of the aromatic side chain and formation of new peptide linkages between subunits.

Spontaneous polymerization of the β-lactam and other peptide antibiotics has already been reported,[1,2,3,4] and has been related to the biological activity—antimicrobial and immunogenic—of these antibiotics which include the natural and semi-synthetic penicillins and cephalosporins.[5] The only member of this series which has received detailed attention as to its probable ordered polymeric structure is benzylpenicillin.[6,7] We are reporting here experiments which assist in determining the polymeric structure of ampicillin, the highly-reactive D(—) epimer of the α-amino derivative of benzylpenicillin.

1. Experimental

Most of the methods used in these experiments have been described previously.[2,3,7,8] Briefly, they consist of the following:

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FRACTIONATION OF ANTIBIOTICS BY

A. Chromatography in Sephadex G-25 columns, the eluates being pooled according to their homogeneity by ultraviolet spectroscopy over the range 340–200 mμ then lyophilized (Fig. 1). This method separated molecules in the molecular weight range less than 1,000 to greater than 5,000.

B. Dialysis to exhaustion in Visking tubing against distilled water. The dialysate yielded molecules of molecular weight less than 5,000; molecules of molecular weight greater than 5,000 were obtained from the retentate.

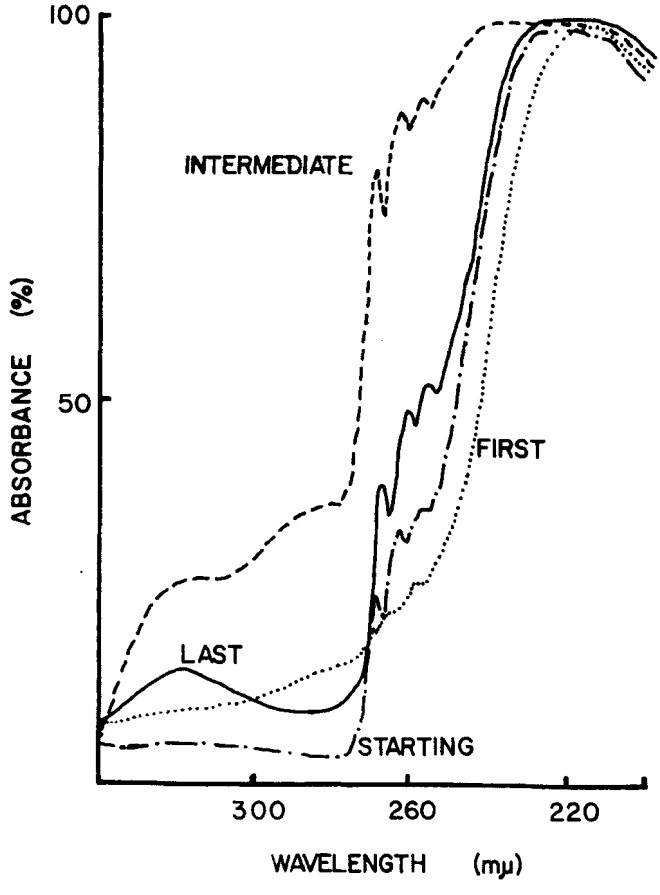


Figure 1. UV absorption spectra of ampicillin fractions.

C. Ultrafiltration using an Amicon Diaflo Cell, with a membrane having an average retention of 1,000 molecular weight, under 100 lb/sq in. pressure of nitrogen. The filtrate contained molecules of less than 1,000 molecular weight, larger molecules being recovered from the cell.

The fractions thus obtained were tested for antimicrobial potency, immunogenicity in animals, and by conventional chemical techniques. Fractions were also examined spectroscopically in the ultraviolet, infra-red and by nuclear magnetic resonance at 60 MHz.

2. Results

Fractionation: Column chromatography yielded 6 fractions (Table 1). Fraction I with a K_{av} approaching zero (MW $\geqslant$ 5,000) is present in small amounts only, as is the last fraction (VI). Fraction V, having a K_{av} approaching one (MW $\leqslant$ 1,000) constituted 45% of the material recovered and is considered to be mono- or dimeric ampicillin (Fig. 1).

Antimicrobial Activity: When the fractions were examined microbiologically, fractions I and II showed only trace antimicrobial activity while Fraction III showed marked activity (40% of the activity of the starting material). Fractions IV and V approached, but did not attain, full potency. The final fraction showed high activity also but less than the two previous fractions (Fig. 2).

Tests for Degradation Products: Fractions were tested for iodine uptake[9] before hydrolysis as a measure of free penicilloate present and after hydrolysis as a measure of intact β-lactam structure. On hydrolysis, the β-lactam ring is opened, yielding penicilloate (Fig. 2). As a more specific test, mercuric chloride titrations were performed.[10] This method utilized the reaction of mercuric chloride with penicilloate to yield penamaldate which was measured by absorption at 285 mμ. The amount of free penamaldate present was measured by reading at 285 mμ both before hydrolysis and before the addition of mercuric chloride (Fig. 2). Mercuric chloride titrations correlated well with the iodometric assay. Subtraction of penamaldate and free penicilloate from the penicilloate present after hydrolysis (total penicilloate) was an indication of the intact β-lactam structure present. It will be seen that these results, in general,

TABLE 1. Properties of Fractions from Ampicillin by Column Chromatography in Sephadex G–25

Fraction	Estimated Mol. wt.	Weight yield (% of starting material)	UV absorbance (mμ) at 322 Penicillenate	285 Penamaldate	$HgCl_2 \rightarrow 285$ Penicilloate	β-lactam IR (~1760 CM^{-1})	β-lactam NMR (~4.5τ)
Ampicillin†	349	100.00	—	—	—	+ +	+ +
I	> 5000	0.38	—	+	+	—	Trace
II	4000	2.86	—	+	+	±	±
III	3000	12.93	—	+	+	+	+
IV	2000	30.65	Trace	—	—	+ +	+ +
V	<1000	45.18	+	—	—	+ +	+ +
VI	<1000	0.20	Trace	—	+	+	+
Total		92.20					

† Ampicillin (U.S.P.) starting material, before fractionation.

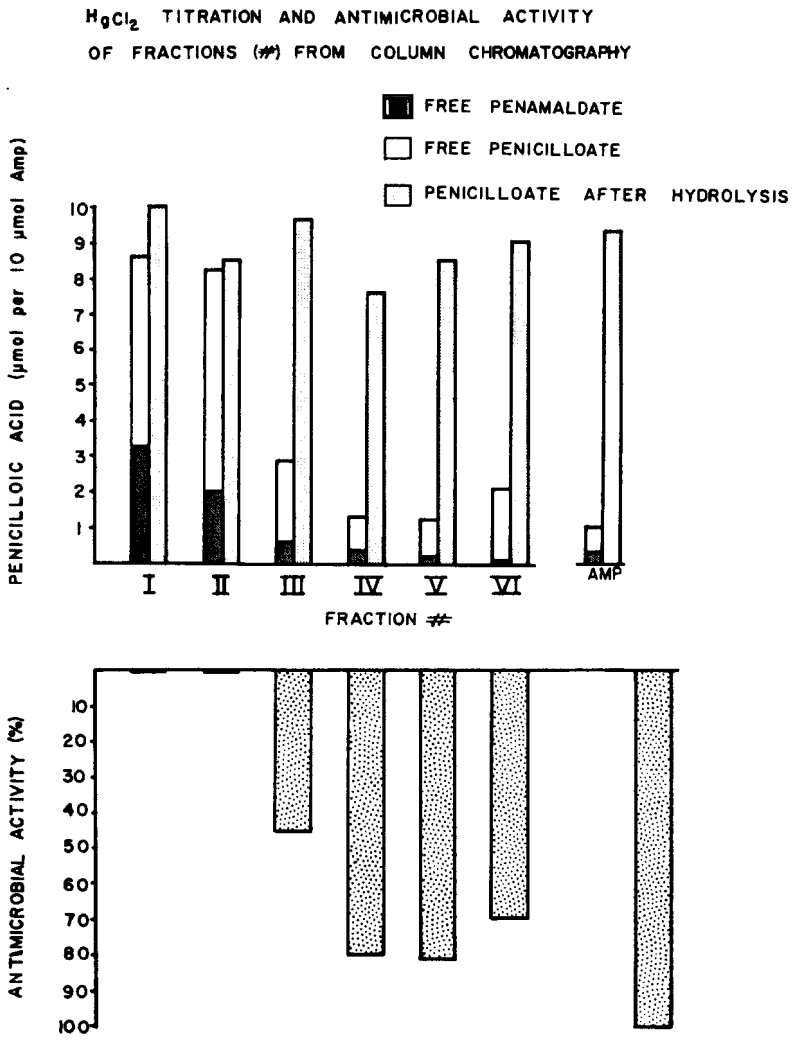


Figure 2. Activity of α-aminobenzylpenicillin polymers.

showed good correlation with the antimicrobial potency tests which were also a measure of intact β-lactam.

3. NMR and IR Spectroscopy

The tentative assignments for the peaks in the NMR spectra

some of which are reproduced in Fig. 3, are shown in Table 2. Fraction V, of lowest molecular weight, thought to be monomeric or dimeric, gave the sharpest NMR and IR spectra. In the NMR, a strong sharp " doublet " centered at 8.47τ was assigned to the methyl groups attached to the thiazolidine ring. The splitting of this singlet peak was considered to be due to the asymmetry of the environment

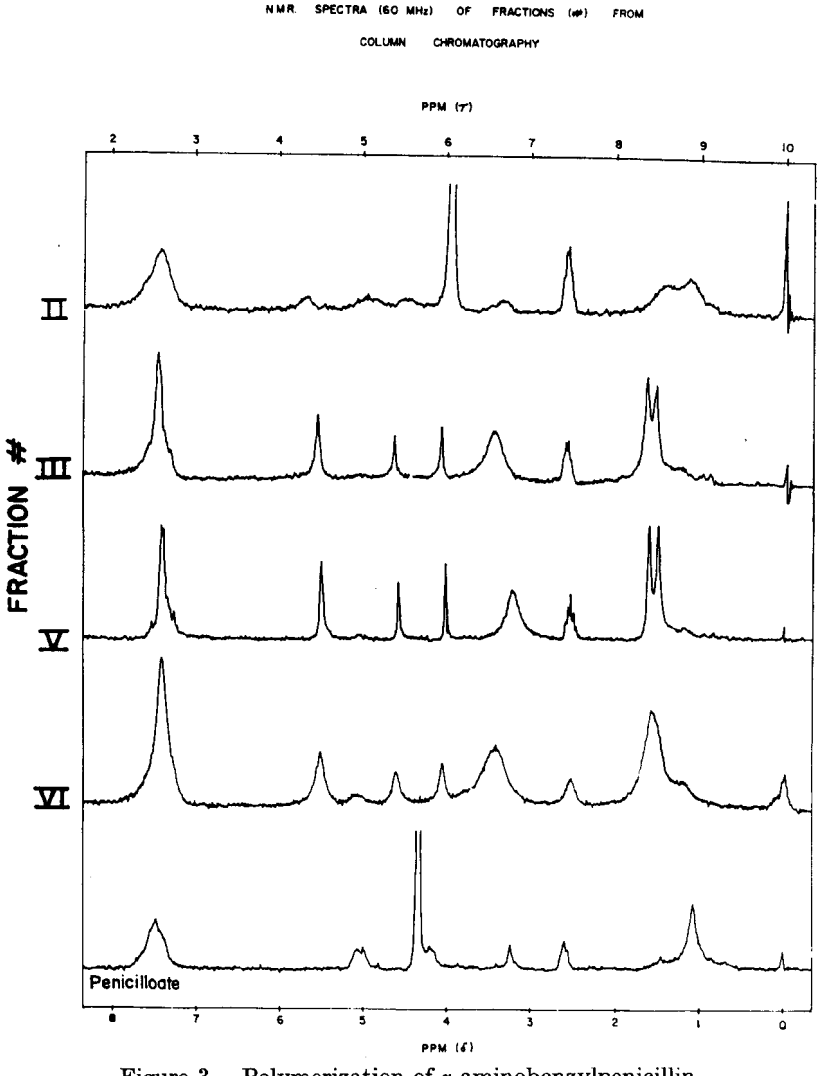
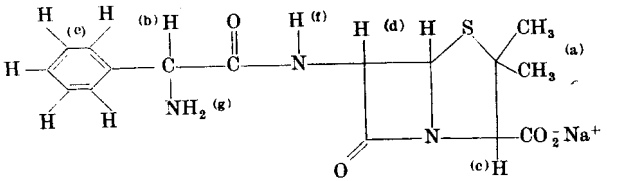


Figure 3. Polymerization of α-aminobenzylpenicillin.

TABLE 2 Polymerization of α-Aminobenzylpenicillin (Ampicillin): NMR Spectra of Fractions (♯) from Column Chromatography (tentative assignments)



Fraction and Solvent	a τ	a Signal†	b τ	b Signal	c τ	c Signal	d τ	d Signal	e τ	e Signal	f τ	f Signal	g τ	g Signal	other τ	other Signal
♯ I	8.7	2S	5.9	?		?	4.5	S	2.54	S	—	—	—	—	6.6	S
D_2O						§										
♯ II	8.8	2S		?	5.6	S	4.32	S	2.68	S	—	—	—	—	6.7	S
DMSO + D_2O	8.6			§											5.1	S
♯ III	8.46	2S	5.95	S	5.39	S	4.47	S	2.55	S	1.2	B	6.58	S B		
DMSO																
♯ IV or V	8.47	2S +	5.99	S	5.42	S	4.50	S	2.60	S	—	—	6.78	S B		
DMSO																
♯ IV or V	8.60	2S +	5.81	S	5.33	?	4.54	S	2.58	S	—	—	—	—		
D_2O						§										
♯ VI	8.42	S +	5.96	S	5.40	S	4.49	S	2.60	S	—	—	6.6	S B	4.92	S
DMSO																
PCO‡	8.92	S +	5.80	S	5.66	?	4.97	D	2.51	S					6.78	S
DMSO + D_2O						§										

† S = Singlet; D = Doublet; + = Additional absorption nearby; B = Broad.
‡ PCO = α-aminobenzylpenicilloic acid.
§ = Water peak and other absorption.

of the methyl groups which are on opposite sides of the ring. Even in this purified material, a small amount of absorption was seen upfield of these peaks. This was probably due to contaminating compounds containing open β-lactam and/or open thiazolidine ring structures. The typically broad peak due to —NH absorption was seen at 6.78τ. This peak, assigned to the α-amino group disappeared upon addition of deuterium oxide (D_2O) to the solution as there was rapid hydrogen-deuterium exchange. The dimethylsulfoxide (DMSO) signal was found near 7.5τ. The singlets at 5.99, 5.42, 4.50 and 2.60τ arose from the benzylic hydrogen, the thiazolidine ring hydrogen, the β-lactam hydrogens and the phenyl hydrogens respectively. Some fine structure was seen on the phenyl peak. The integration of the spectrum, assuming 5 H's for the phenyl absorption, fitted very well except for only 5.5 H's instead of 6 in the methyl absorption. The infra-red spectrum was similar to starting material in showing distinctive β-lactam absorption at 1760 cm^{-1} and a relatively clear fingerprint region although not as sharp as that of the starting compound prior to chromatography. All IR spectra were read in potassium bromide in the solid state. Fraction IV closely resembled Fraction V but did contain trace amounts of small polymer.

The early fraction labeled Fraction I in Table 2 appeared to be very polymeric with a molecular weight in excess of 5,000. Its NMR spectrum in DMSO showed the broad, poorly defined peaks characteristic of some polymeric compounds. Addition of D_2O to the DMSO solution increased the sharpness of the peaks, due perhaps to a decrease in viscosity or to a precipitation of some of the polymer. Some solid material was seen to come out of solution upon addition of D_2O. The integration of the curve was of no assistance due to the poor resolution of the peaks. The IR spectrum was poorly resolved and showed no β-lactam absorption near 1760 cm^{-1}. The fingerprint region showed very few peaks and was decidedly changed as compared with starting material or monomer.

Fraction II, of somewhat lower molecular weight, again showed the broad, ill-defined peaks which clarified somewhat upon addition of D_2O to the DMSO solution. The peaks were clearer than in Fraction I as the material was less polymeric and showed two distinct absorptions in the methyl region near 8.6 and 8.8τ. The downfield peak was

thought to be due to the methyls on the thiazolidine ring. In other spectra of lower molecular weight material, this 8.6τ peak was the one resolved into equal peaks probably arising from this methyl group asymmetry. Whether the higher field (8.8τ) peak arose from a methyl or methyls in a different environment, due to polymer formation and/or to opening of the thiazolidine or β-lactam ring was not clear. A small peak was seen in the region of 4.5τ assigned to the hydrogens on the β-lactam ring. There was also a broad peak upfield of this near 5.1τ which corresponded to a peak found in the penicilloic acid derivative. This absorption probably arose from the same two hydrogens on the compound having the β-lactam opened. A small amount of β-lactam absorption at 1760–1770 cm^{-1} was seen in the IR spectrum. The fingerprint region was somewhat clearer than that seen in the heavier polymer but still had few peaks and bore little resemblance to that of the monomer. This type of blurred, poorly resolved spectrum is sometimes seen in mixed or impure polymeric materials.

Fraction III gave a much clearer, well-defined NMR spectrum in DMSO as the molecular weight was further decreased. The methyl absorption began to show a " doublet " with center at 8.5τ with some irregular absorption upfield. The broad α-amino signal appeared near 6.6τ. Not illustrated, but seen in the spectrum, was a very broad, low peak near 1.2τ arising from the amide hydrogen. This peak was not seen in the presence of D_2O. The IR began more closely to resemble that of the monomer with its strong β-lactam absorption and a more distinctive fingerprint.

Fraction VI, which followed the monomer, showed a return to polymeric-type material. The NMR spectrum again showed loss of clarity, broadening of the peaks and base line absorption near the peaks. There was still strong absorbance in the β-lactam region but an upfield peak at 4.92τ was present. The IR confirmed the presence of β-lactam but showed a less resolved fingerprint region with similarity to, but by no means identity with, earlier polymers. The NMR also showed the growth of absorption upfield from the methyl absorption which appeared then as a singlet. This coalescence of the methyl " doublet " might be partially due to poor resolution caused by viscosity as, on addition of D_2O, a slight splitting of the peak was seen.

Comparison of the NMR and IR spectra of α-aminobenzylpenicilloic acid with the others showed some interesting parallels. This acid, obtained by hydrolysis by enzyme in whole cells of ampicillin-resistant *E. coli*, appeared polymeric and in physical appearance resembled the very fluffy early fractions of high molecular weight. Addition of D_2O greatly improved the spectrum which in pure DMSO showed only three extremely broad absorption regions. Decided shifts in peak positions were seen, compared with Fraction V. The absorption at 4.97τ, a poorly resolved doublet, was almost certainly due to the hydrogens on the opened β-lactam. Traces of this absorption could be seen in all fractions, even faintly in the monomer. There was a strong methyl peak with side bands upfield at 8.92τ. This might have accounted for some of the upfield absorption in the polymeric material. The IR confirmed the total loss of the β-lactam ring and showed some resemblance to the fingerprint region of the polymeric early fractions and less resemblance to the later polymers.

The NMR and IR spectra of the fractions therefore confirm the results obtained by assay for antibacterial activity and by penicilloate titration (Fig. 2) but, only in the monomer, has integration of the NMR spectrum proved very useful. The other fractions gave partial numbers of hydrogens for other peaks. This leads to the conclusion that there are mixtures of compounds or at least of penicillin residues in the samples. The shift of the $—NH_2$ peak in the monomer compared to the polymers is not understood but small changes in environment are known to cause shifts in these absorptions. It is interesting to note that the hydrogens on the β-lactam had the same chemical shift considering the differences in their neighboring groups.

4. Discussion

These results show that pure ampicillin (MW = 349) in aqueous solution forms macromolecules with weights up to and exceeding 5000. The proportion of heavy macromolecules (MW $\geqslant$ 5000) is low (approximately 0.4% of starting material by weight), of intermediate compounds (MW = 1000–5000) much higher. Titrations of the fractions obtained by column chromatography (Table 1, Fig. 2) show that the formation of the larger molecules is associated with opening

of the β-lactam ring to yield free α-aminopenicilloic and penamaldic acid, with a corresponding loss in antimicrobial potency. Conversion of ampicillin to its penicilloic acid derivative by alkaline hydrolysis yields a product showing polymeric properties on IR and NMR spectroscopy. Penicilloic acid therefore seems to play a key part in all sizes of polymer but there are other differences between the polymers. β-lactam structure is virtually absent in the heavy polymer and there is a shift in the NMR spectrum of the thiazolidine methyl groups The α-amino is also relatively inconspicuous though this may be due to technical factors. The polymer is fluffy, practically devoid of antibacterial activity and would seem therefore to be composed of subunits of α-aminopenicilloic acid joined either at the lactam carbonyl, the N_4 or the α-amino group (Fig. 4).

The intermediate products show some preservation of β-lactam structure, of dimethyl protons, of phenyl protons and of the α-amino signal. These polymers would appear, therefore, to be composed of 3–10 subunits of intact ampicillin and its penicilloic acid derivative, though the possibility that other degradation products are present cannot be excluded. Possible sites of covalent linkage leading to polymers of the various sizes encountered in our study are indicated in Fig. 4, from which it will be seen that, as polymerization proceeds, the likelihood of mixed polymers or aggregates increases. In support of this is the indication of repolymerization of the small molecules of ampicillin and its degradation products seen in the final fraction (VI) separated by column chromatography (Fig. 3).

An earlier study of the polymers of benzylpenicillin[2] showed that penicillenic acid was a key degradation product and subunit of a disulfide polymer, though the formation of linear polymers from repeating subunits of penicilloic acid was also described.[7] Ampicillin, in contrast, seems to polymerize differently, with little or no dependence upon the liberation of the penicillenic acid derivative. In both penicillins, other potentially reactive though weak groups, such as the ring carboxyl, do not seem to be involved.

Biosynthesis of the dipeptide β-lactam antibiotics involves cyclic fusion of two or more amino-acids; polymerization, as described above, appears to depend upon some loss of cyclic structure and to lead to ordered structures resembling oligopeptide chains.

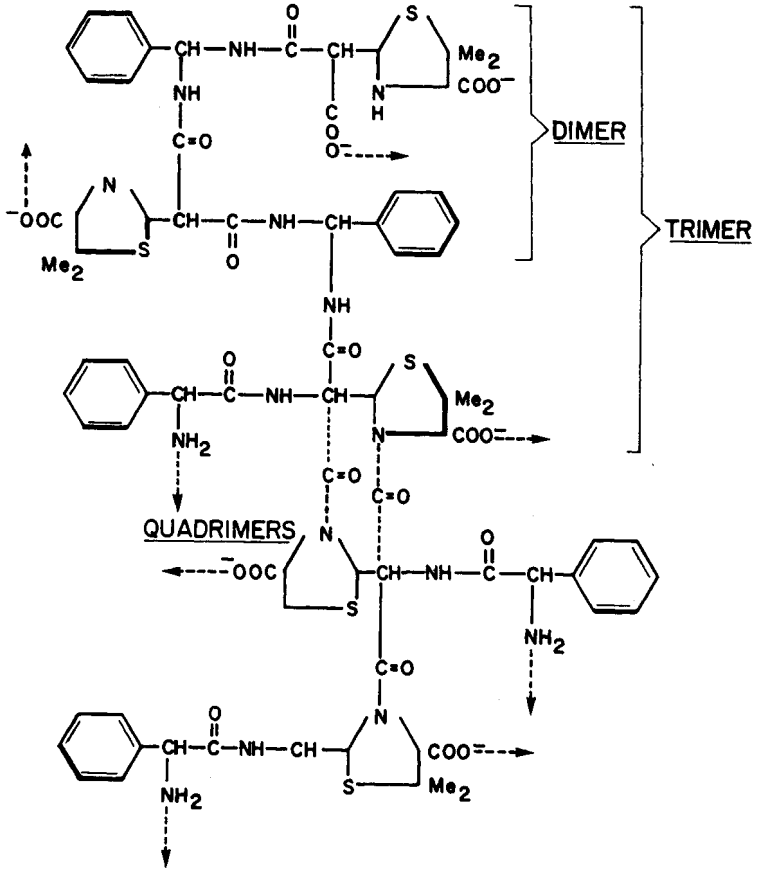


Figure. 4 Polymers of α-aminobenzylpenicillin (ampicillin).

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