Journal of Chromatography, 132 (1977) 51–59

CHROM. 9587

POLYMERIZATION OF PENICILLINS

IV. SEPARATION, ISOLATION AND CHARACTERIZATION OF AMPICILLIN POLYMERS FORMED IN AQUEOUS SOLUTION

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(Received June 16th, 1976)

SUMMARY

Polymeric substances formed from ampicillin sodium in aqueous solution have been separated according to charge by anion-exchange chromatography on a column of DEAE-Sephadex A-25 using a linear sodium chloride gradient at a constant pH of 7.4. The separated materials, ranging in size from the monomer to the octamer, have been characterized by various functional-group analyses. A homogeneous well-defined ampicillin dimer, tetramer and hexamer have been isolated from the effluent in high yields. The separation and isolation of the ampicillin polymers permit a conclusive establishment of their structures, which in turn has confirmed previous suggestions that the polymers are formed through a chain process involving aminolysis of ampicillin at the β-lactam carbonyl moiety by the amino group of the side-chain of a second ampicillin molecule.

INTRODUCTION

The formation of higher-molecular-weight substances in aqueous solutions of ampicillin sodium [D(−)-α-aminobenzylpenicillin sodium] on storage for a few days at room temperature has been observed by several workers[1–9]. The substances, so-called ampicillin polymers, are assumed to be formed through a chain process involving nucleophilic attack of the amino group of the side-chain in one ampicillin molecule on the reactive β-lactam moiety of a second molecule[4,5,9–11]. The structure of such ampicillin polymers depicted in Fig. 1 was proposed by Grant[4] and has been supported by other workers[5,9,10,12]. However, the structure remains to be definitely established. Up to now the detection and isolation of the polymeric substances have been accomplished either by gel filtration or by dialysis, but neither technique has permitted a separation of the individual polymers or made obtainable pure well-defined polymers. Interest in the polymerization of ampicillin originates principally from the finding[6,7,13] that the polymerization products possess strong antigenic properties, as shown in animal experiments, and therefore may play a part in the elici-

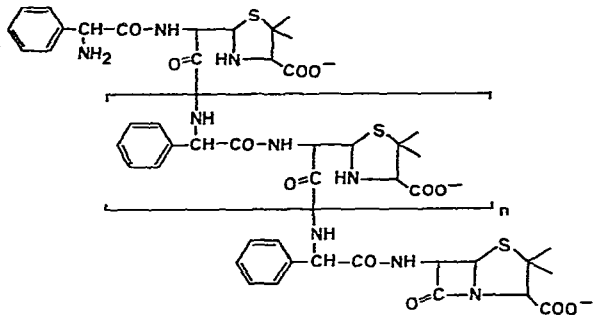


Fig. 1. Structure of the polymers of α-aminobenzylpenicillin (ampicillin).

tation of some clinical allergic reactions to ampicillin. In addition, di- and polymerization have been shown[11] to account for the marked concentration-dependence of the stability of ampicillin sodium in aqueous solution.

Inspection of the suggested structure of the polymeric substances shows that, besides differing in molecular weight, the various polymers differ with respect to their overall charge at certain pH values. Whereas all of the substances contain one free primary amino group per molecule, the number of carboxyl groups increases proportionately with increasing degree of polymerization. Thus, at pH values where the acid groups are ionized, the various polymers are characterized by an increasing negative overall charge with increasing chain length. This difference in charge led us to suggest that fractionation of ampicillin, its dimer and higher polymers might be more practicable by use of a technique based on anion-exchange chromatography (AEC). In this paper we present conditions for an efficient separation of ampicillin di- and poly-mers by AEC on DEAE-Sephadex A-25. The separation obtained has for the first time made possible the isolation of essentially homogeneous well-defined polymers and prepared the way for a conclusive establishment of their structures.

MATERIALS AND METHODS

Apparatus

Ultraviolet and visible spectral measurements were performed by use of a Zeiss PMQ II spectrophotometer and a Perkin-Elmer 124 recording spectrophotometer, with 1-cm cuvettes. Infrared spectra were recorded for potassium chloride discs on a Unicam SP 200 spectrophotometer. pH values were measured on a Radiometer Type PHM 26 instrument.

Chemicals

Ampicillin sodium (Doktacillin®) was purchased from AB Astra, Södertälje, Sweden. DEAE-Sephadex A-25 (capacity, 3.5 mequiv./g; particle size, 40–120 μm) was obtained from Pharmacia, Uppsala, Sweden. 2,4,6-Trinitrobenzenesulphonic acid was purchased from Sigma, St. Louis, Mo., U.S.A. All of the other chemicals used were of reagent grade.

Formation of ampicillin polymers

Ampicillin sodium was dissolved in distilled water to give a concentration of

20%. After adjustment of the pH to 8.5 ± 0.1 with a few drops of 2 *M* hydrochloric acid, the solution was allowed to stand at room temperature (23 ± 2°) for from one to several days. Based on the results of a previous kinetic study[11], a pH of *ca.* 8.5 was assumed to be optimal for the formation of polymers at the expense of the concomitant hydrolysis of ampicillin, account being taken of the decreased solubility of ampicillin at lower pH values. The pH of the solution decreased during storage but no precipitate appeared even after storage for 2 weeks.

Ion-exchange chromatography

The fractionation of the aged aqueous solutions of ampicillin sodium was carried out at ambient temperature on a column (36 × 2.6 cm) filled with DEAE-Sephadex A-25 gel. Before use, the anion-exchanger was swelled in the starting buffer for 3 h on a steam-bath; the buffer solution used was 0.05 *M* phosphate (pH 7.4) containing sodium chloride (0.2 *M*). After sedimentation and removal of the supernatant, the ion-exchanger was washed with the buffer solution until the pH of the washings stabilized at 7.4. Regeneration of the ion-exchanger after a run was similarly accomplished by repeated washing with the starting buffer.

The elution of a portion, usually 1 ml, of the solution of polymerized ampicillin sodium applied to the column was achieved with a linear sodium chloride gradient at a constant pH of 7.4 maintained by use of a 0.05 *M* phosphate buffer. The linear gradient was produced by means of a constant-speed peristaltic pump having three channels, as described by Ayad *et al.*[14]. The mixing beaker initially contained 1500 ml of 0.05 *M* phosphate–0.2 *M* sodium chloride solution (pH 7.4) and the reservoir contained 0.05 *M* phosphate–1.75 *M* sodium chloride solution (pH 7.4). A flow-rate of 67–70 ml/h was maintained, and the absorbance of the effluent was monitored continuously at 260 nm (or at 270 nm when a 10-ml sample was applied to the column) on a spectrophotometer connected to a potentiometric recorder. After passage through the spectrophotometer cuvette, the effluent was collected in fractions of 10 ml.

Isolation of ampicillin di-, tetra- and hexa-mers

A 10-ml portion of a 20% aqueous solution of ampicillin sodium (initial pH 8.5), which had been kept at 20–25° for 3 days, was applied to the column and eluted as described above. Fractions which (see below) were shown to contain a pure dimer, tetramer and a hexamer, respectively, were pooled and treated as follows.

After adjustment of the pH to 5.5 with 2 *M* HCl, the solution of the dimer (82 ml; $A_{260} = 4.0$, where A_{260} is the absorbance at 260 nm) was concentrated *in vacuo* at 20° to *ca.* 20 ml. The concentrated solution was cooled to 0–5° and adjusted to pH 3.1 with 2 *M* HCl. A white precipitate formed and was filtered off, washed thoroughly with cold water and finally dried *in vacuo* (20°, 18 h) over phosphorus pentoxide. 320 mg were obtained, corresponding to 71% of the total amount contained in the treated fractions.

The solution (78 ml, $A_{260} = 2.1$) containing a tetramer was cooled to 0–5° and adjusted to pH 3.0 without the preceding concentration step. The precipitate was filtered off and washed and dried as described for the dimer, 205 mg were collected, corresponding to a yield of 88%.

The precipitate formed by adjusting the pH of pooled fractions containing a hexamer (90 ml, $A_{260} = 1.5$) to 3.0 was very fine and could not be collected by filtra-

tion. However, the addition of 15 ml of ethyl acetate to the acidified and cooled solution afforded an immediate agglutination of the hexamer, which could then easily be isolated by filtration. After washing and drying, 170 mg of the compound were collected, corresponding to a yield of 86%. The addition of ethyl acetate was also found to be necessary in order to isolate the hexamer from a solution which had been concentrated to *ca.* 20 ml prior to acidification.

Analytical characterization of the separated and isolated substances

The effluent appearing within separated peaks as well as aqueous solutions (0.05 *M* phosphate, pH 7.4) of the isolated di- and polymers were analyzed for phenyl, primary amino, penicilloamide and penicilloate groups, and for intact β-lactam rings as follows.

Phenyl groups. Phenyl groups were determined by measuring the absorbance at 260 nm. Since 6-aminopenicillanic acid exhibits only a very small absorption at 260 nm compared to ampicillin, and since penicilloyl derivatives of ampicillin, such as α-aminobenzylpenicilloic acid and N-(α-aminobenzylpenicilloyl)glycine (these compounds were prepared as previously described[11]), were shown to exhibit exactly the same molar absorptivity as ampicillin (and as benzylpenicillin) at this wavelength, the absorbance at 260 nm is assumed to be a reliable measure of the concentration of phenyl groups in the di- and polymers. This was substantiated by the similarity of the UV spectra of the polymers to that of ampicillin in the range 250–330 nm. For the calculation of the concentration of phenyl groups, a molar absorptivity of 240, as found for ampicillin, was used. All of the absorbance measurements were made in 0.05 *M* phosphate buffer (pH 7.4).

Primary amino groups. Primary amino groups were quantitated by using the colorimetric 2,4,6-trinitrobenzenesulphonic acid assay of Satake *et al.*[15] with a modification previously described[16]. A reaction time of 30 min at pH 7.6 and room temperature was sufficient to complete the trinitrophenylation of the primary amino groups in each compound. A molar absorptivity of $11.9 \cdot 10^3$ (420 nm) was used for the calculations. This value was determined with ampicillin sodium.

Penicilloyl and penicilloate groups. Penicilloamide and penicilloate groups were determined by means of the spectrophotometric penamaldate assay[17] as modified by Schwartz and Delduce[18]. This assay (treatment of solutions at pH 7 with mercuric chloride and subsequent measurement of the absorbance and absorbance stability at 285 nm) is specific to penicilloic acids and penicilloyl amines, and permits distinction between them. For the calculation of the concentration of penicilloamide and penicilloate groups, molar absorptivities of $21.5 \cdot 10^3$ and $9.35 \cdot 10^3$, respectively, were used[11].

Intact β-lactam moieties. These groups were determined by using the spectrophotometric method of Bundgaard and Ilver[19]. A molar absorptivity of $27 \cdot 10^3$ (325 nm)[19] was used in calculation of the concentrations. A blank was prepared by mixing equal volumes of the solution to be assayed and of 2 *M* sodium hydroxide. After standing for 10 min at room temperature, the mixture was neutralized with 2 *M* HCl and then treated as was the unhydrolyzed solution. The latter was diluted to the same extent as the blank, but with water. For the determination of the β-lactam moiety of ampicillin, the spectrophotometric assay was used in a modified form as reported previously[20]. For the isolated polymers, the intact β-lactam structure was examined further by IR spectroscopy.

All of the analyses were made at least three times, the deviation between the individual determinations being less than 5%.

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows the elution pattern of 1-ml portions of a degraded ampicillin sodium solution obtained on a DEAE-Sephadex A-25 column using a linear sodium chloride gradient at a constant pH of 7.4. Several experimental conditions other than those used in Fig. 2 were tried, *e.g.*, elution at a constant ionic strength of 0.4 or 0.6 *M*, but they gave a poorer resolution. With the selected system, at least 10 ml of a degraded 20% ampicillin sodium solution could be fractionated with a degree of resolution as good as that obtained with a 1-ml sample.

A chromatogram developed immediately after the preparation of the ampicillin

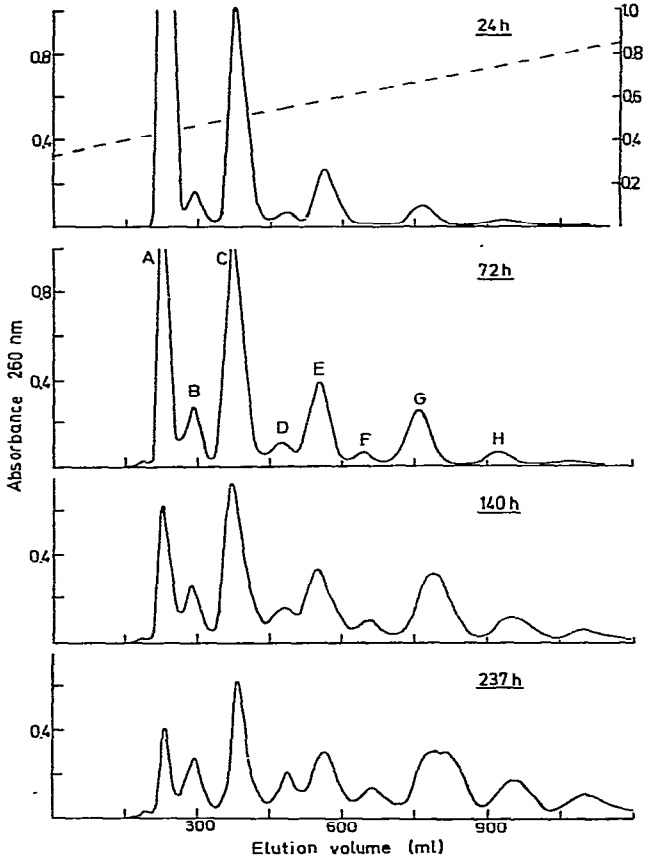


Fig. 2. Fractionation by anion-exchange chromatography of 1-ml portions of a 20% aqueous solution of ampicillin sodium (initial pH 8.5) kept at room temperature for the specified periods. Column (36 × 2.6 cm I.D.) packed with DEAE-Sephadex A-25. Eluent, 0.05 *M* phosphate (pH 7.4), with a linear sodium chloride gradient. Flow-rate, 67 ml/h. The right ordinate represents the ionic strength (*M*) of the effluent.

sodium solution showed only one peak (peak A in Fig. 2). On ageing, the penicillin solution gave a series of successive peaks, A to H, as shown in Fig. 2. Whereas peak A continued to decrease in size with time, peak C at first increased and then decreased. Peaks G and H increased slowly with time on storage. After storage for 5–6 days, the ampicillin solution gave an additional late-eluting peak which increased in intensity with further storage. Fig. 2 shows also that the resolution and the sharpness of some of the peaks became poorer with increasing time of storage of the polymer solution. This is ascribed to the hydrolytic opening of the β-lactam rings in the polymers, resulting in an overlap of peaks between an *n*-mer and an (*n*—1)-mer with an opened β-lactam ring.

At constant flow-rate the elution volumes of the separated peaks were highly reproducible. The elution volumes obtained in six runs agreed within ± 2.6%.

Peak identification

The collected peaks were identified by analysis of the various functional groups occurring in the postulated polymer structure shown in Fig. 1. Fractions from the middle of the peaks appearing in a chromatogram of a 1-ml sample of a 3-days old ampicillin sodium solution gave the results summarized in Table I. In the following discussion, *R* describes the ratio of the molar concentrations of the primary amino to the phenyl to the β-lac am to the penicilloamide to the penicilloate groups.

TABLE I

ANALYSIS OF COLLECTED PEAK FRACTIONS FOR VARIOUS FUNCTIONAL GROUPS

After standing at room temperature for 3 days, 1 ml of 20% aqueous solution of ampicillin sodium (initial pH 8.5) was chromatographed as described in the text.

*Peak**	*Elution volume (ml)*	*Molar concentration · 10³*					*R***
		NH_2	*phenyl*	*β-lactam*	*penicilloamide*	*penicilloate*	
A	233	1.88	1.82	1.84	0	0	1.0:1.0:1.0:0:0
B	293	1.00	1.1	0.04	0	1.14	1.0:1.1:0.0:0:1.1
C	378	1.80	3.75	1.87	2.09	0	1.0:2.1:1.0:1.1:0
D	480	0.38	0.94	0.14	0.54	0.25	1.0:2.5:0.4:1.4:0.7
E	555	0.75	2.89	0.77	2.24	0	1.0:3.9:1.0:3.0:0
F	663	0.11	0.50	0.06	0.37	0.05	1.0:4.5:0.5:2.7:0.5
G	775	0.31	1.87	0.31	1.56	0	1.0:6.0:1.0:5.0:0
H	1105	0.06	0.47	0.07	0.41	0	1.0:7.8:1.2:6.8:0

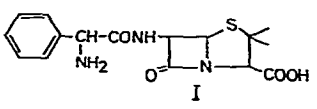
* The peak designation refers to that shown in Fig. 2.

** Defined as the ratio [NH_2]:[phenyl]:[β-lactam]:[penicilloamide]:[penicilloate]. In the calculation of this ratio [NH_2] was set equal to one.

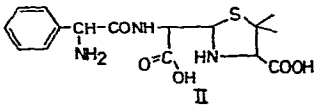
Peak A. This is due to ampicillin (I) since the peak was eluted at the same volume as ampicillin and showed an identical *R* value to that of ampicillin.

Peak B. This peak represents the penicilloic acid of ampicillin (II). An authentic sample of α-aminobenzylpenicilloic acid, prepared[11] by hydrolysis of ampicillin in 1 *M* NaOH, had an elution volume corresponding to that of peak B. In addition, the *R* value obtained was similar to that calculated for α-aminobenzylpenicilloic

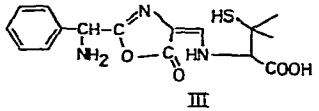
acid. The UV spectrum of the peak revealed the presence of a minor component with an absorption maximum at 320 nm. This compound is probably α-aminobenzylpenicillenic acid (III), its disulphide or a higher sulfhydryl-oxidation product.



I



II



III

Peak C. The functional-group analysis gave an R value similar to that expected for a dimer with the structure indicated in Fig. 1 ($n = 0$). The zero value for penicilloate, together with the agreement between the calculated and found values for β-lactam groups, show that the β-lactam ring in the dimer is not opened.

Peak D. The analytical data show that this minor peak is due to a mixture of two compounds. The data are compatible with a mixture of a trimer ($n = 1$) having a closed β-lactam ring and a dimer ($n = 0$) having an open β-lactam ring in the molar ratio 1:1.8. Since these substances each contain one amino group and three carboxylate groups, their elution at the same volume is to be expected.

Peak E. This peak represents a tetramer ($n = 2$) with an intact β-lactam ring in the terminal unit, as shown by the analytical results.

Peak F. As is the case for peak D, the analytical data for peak F indicate the presence of two substances. On the basis of its relative elution position, the peak is assumed to be due to a tetramer with an open β-lactam ring and to a pentamer ($n = 3$) with an intact β-lactam ring. The R value obtained is in accord with the presence of these substances in a molar ratio of 1:1.2 (open tetramer to closed pentamer).

Peak G. The analytical results obtained for this peak are in complete agreement with data calculated for a hexamer ($n = 4$) of the structure shown in Fig. 1.

Peak H. The R value for this peak indicates an octamer ($n = 6$) with a closed β-lactam ring in the terminal unit.

The results of a similar analytical characterization of the isolated substances obtained by chromatography of a 10-ml portion of a 3-days old ampicillin solution (see Experimental section) are given in Table II. The data show that the compounds are essentially homogeneous and have been isolated without the occurrence of any structural changes. In addition to this functional-group characterization, the isolated solids were examined by IR spectroscopy. The only difference in the spectra of the

TABLE II

FUNCTIONAL-GROUP ANALYSIS OF ISOLATED AMPICILLIN POLYMERS

Substance	*R**	
	Found	*Calc.***
Dimer	1.0:2.0:1.0:1.1:0.0	1:2:1:1:0
Tetramer	1.0:4.0:1.0:3.1:0.0	1:4:1:3:0
Hexamer	1.0:6.1:1.1:5.0:0.0	1:6:1:5:0

* Defined as in Table I.

** On the basis of the structure shown in Fig. 1.

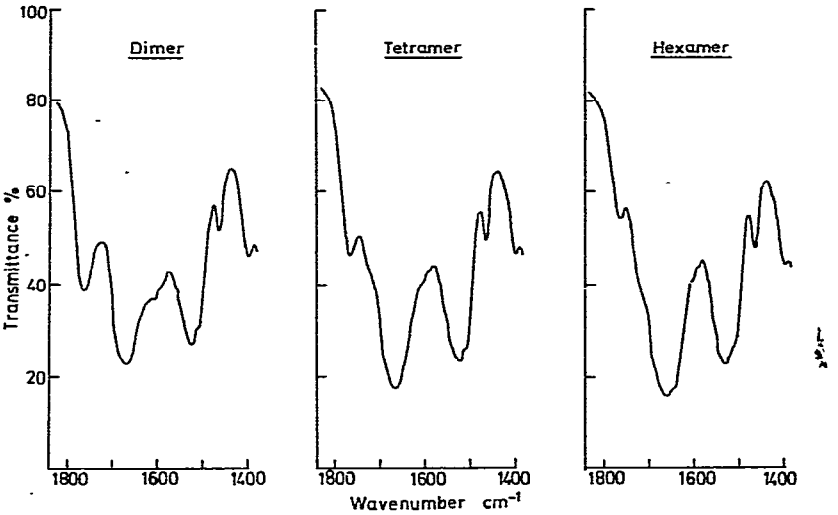


Fig. 3. IR spectra of the isolated ampicillin polymers.

three compounds occurred at 1765 cm^{-1} (β-lactam absorption) where the absorption decreased with increasing degree of polymerization (Fig. 3). The spectra were identical to that of ampicillin sodium, apart from bands in the spectrum of the latter at 1410 and 1605 cm^{-1} due to the ionized carboxylic acid function.

In connection with the kinetic arguments, advanced in a previous paper[11], for the assignment of the structure shown in Fig. 1 to ampicillin di- and poly-mers formed spontaneously in concentrated aqueous solutions, the results of the analysis of the separated polymers as described in this paper provide a conclusive confirmation of this structure. In addition, the order of emergence of the di- and poly-mers in the effluent from the anion-exchanger follows the order to be expected from the difference in the number of negatively charged groups in the structures. In this connection it should be added that the secondary thiazolidine amino groups in the structures are unprotonated at pH 7.4, since the pK_a of these groups can be estimated[21] to be *ca.* 5.

Finally, it is of interest to note that polymers with an even degree of polymerization, *e.g.*, dimer, tetramer, etc., appear to be formed to a much larger extent than polymers composed of an uneven number of monomeric units (see Fig. 2). An examination of the relative amino and β-lactam reactivity of ampicillin and ampicillin dimer may provide an explanation of this observation.

REFERENCES

1 G. T. Stewart, *Antimicrob. Ag. Chemother.*, (1967) 543.
2 G. T. Stewart, *Antimicrob. Ag. Chemother.*, (1968) 128.
3 G. T. Stewart, B. T. Butcher, S. S. Wagle and M. K. Stanfield, in J. F. Johnson and R. S. Porter (Editors), *Liquid Crystals and Ordered Fluids*, Plenum Publ., New York, 1970, p. 33.
4 N. H. Grant, in B. Weinstein and S. Lande (Editors), *Peptides: Chemistry and Biochemistry*, Marcel Dekker, New York, 1970, p. 487.
5 H. Smith and A. C. Marshall, *Nature (London)*, 232 (1971) 45.
6 J. M. Dewdney, H. Smith and A. W. Wheeler, *Immunology*, 21 (1971) 517.
7 H. Smith, J. M. Dewdney and A. W. Wheeler, *Immunology*, 21 (1971) 527.

8 S. Shaltiel, R. Mizrahi and M. Sela, *Proc. Roy. Soc., Ser. B*, 179 (1971) 411.
9 E. J. Kuchinskas and G. N. Levy, *J. Pharm. Sci.*, 61 (1972) 727.
10 M. A. Schwartz and W. L. Hayton, *J. Pharm. Sci.*, 61 (1972) 906.
11 H. Bundgaard, *Acta Pharm. Suecica*, 13 (1976) 9.
12 B. T. Butcher, M. K. Stanfield, G. T. Stewart and R. Zemelman, *Mol. Cryst. Liq. Cryst.*, 12 (1971) 321.
13 A. C. Munro, J. M. Dewdney, H. Smith and A. W. Wheeler, *Int. Arch. Allergy Appl. Immunol.*, 50 (1976) 192.
14 S. R. Ayad, R. W. Bonsall and S. Hunt, *Anal. Biochem.*, 22 (1968) 533.
15 K. Satake, T. Okuyama, M. Ohashi and T. Shinoda, *J. Biochem. (Tokyo)*, 47 (1960) 654.
16 H. Bundgaard, *Arch. Pharm. Chem., Sci. Ed.*, 4 (1976) 25.
17 C. H. Schneider and A. L. de Weck, *Helv. Chim. Acta*, 49 (1966) 1689.
18 M. A. Schwartz and A. J. Delduce, *J. Pharm. Sci.*, 58 (1969) 1137.
19 H. Bundgaard and K. Ilver, *J. Pharm. Pharmacol.*, 24 (1972) 790.
20 H. Bundgaard, *J. Pharm. Pharmacol.*, 26 (1974) 385.
21 H. D. C. Rapson and A. E. Bird, *J. Pharm. Pharmacol.*, 15 (1963) 222T.