The Structure of Bacilysin and Other Products of *Bacillus subtilis*

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1. Mass spectra of the trimethylsilyl derivative and the methyl ester of the *N*-trifluoroacetyl derivative of bacilysin indicated that the antibiotic had a molecular weight of 270. Several peaks in the spectrum of the methyl ester were consistent with the presence of an *N*-terminal alanine residue in the molecule. 2. The proton-magnetic-resonance spectrum of bacilysin confirmed that the antibiotic contained an epoxide group and the spin-spin splitting of the protons of the epoxide group indicated that the side chain of the epoxycyclohexanone ring was attached at C-4 and was $\alpha\beta$ to the keto group. 3. The formation of an $\alpha\beta$ -unsaturated ketone on reduction of bacilysin with chromous chloride also showed that the epoxide was $\alpha\beta$ to the keto group. 4. The optical-rotatory-dispersion curve of bacilysin showed a positive Cotton effect. On the assumption that the reversed Octant rule for $\alpha\beta$ -epoxyketones was applicable this revealed the absolute stereochemistry and enabled a definitive structure to be assigned to the molecule. 5. Similar measurements showed that substance AA1, isolated from culture supernatants, was the *C*-terminal amino acid of bacilysin. 6. Hydrolysis of substance P2 with leucine aminopeptidase and the mass spectrum of the methyl ester of its *N*-trifluoroacetyl derivative showed that this substance was L-analyt-L-alanine. 7. These results are discussed in relation to the biogenesis of bacilysin.

Earlier studies showed that bacilysin contained an *N*-terminal alanine residue, that it yielded L-tyrosine as well as L-alanine on hydrolysis, and that it contained a keto group and an epoxide group (Rogers, Lomakina & Abraham, 1965). These findings suggested that the antibiotic might be a dipeptide of molecular weight 270 whose *C*-terminal amino acid had a side chain containing an epoxycyclohexanone ring. However, a definitive structure could not be proposed. The molecular formula of bacilysin was not unequivocally established and no information was available about the relative positions of the carbonyl group, epoxy group and side chain in the proposed epoxycyclohexanone ring, or about the stereochemistry of this fragment of the molecule. The present paper describes the application of physicochemical and other methods to the solution of the problems involved and to the determination of the structures of two substances other than bacilysin (substances AA1 and P2) that were obtained from culture fluids of *Bacillus subtilis* A 14 (Walker & Abraham, 1970).

The mass spectrum of the trimethylsilyl derivative of bacilysin contained a peak at *m/e* 416, corresponding to the molecular ion of a compound with mass 270 and with two of its hydrogen atoms

substituted by trimethylsilyl groups. The spectrum indicated that there was contamination with silicone grease, but the latter does not yield a prominent peak in this region (Biemann, 1962). The mass spectrum of a preparation of the methyl ester of the *N*-trifluoroacetyl derivative of bacilysin yielded a peak at *m/e* 380, which was also consistent with a molecular weight of 270 for the antibiotic itself. Thus these results confirmed the earlier tentative suggestion that the molecular formula of bacilysin was $C_{12}H_{18}N_2O_5$ (Rogers *et al.* 1965). The appearance of a peak at *m/e* 394 (380 + 14) in the spectrum of the preparation of the methyl ester of the *N*-trifluoroacetyl derivative could be accounted for by the addition of methanol across the epoxide and the subsequent elimination of water (possibly during volatilization in the spectrophotometer) to yield a vinyl ether.

Peaks at *m/e* 140 and 141 in the main spectrum of the methyl ester of the *N*-trifluoroacetyl derivative of bacilysin correspond to the ion

and a similar ion with one more hydrogen atom respectively, which are typical ions from *N*-acylated peptides with an *N*-terminal alanine residue (Weygand, Prox, Fessel & Sun, 1965;

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Stenhagen, 1961). Peaks at m/e 224 and 210 correspond to positive ions of the fragments

and

respectively. Hence the mass spectrum confirmed that the alanine residue in bacilysin is *N*-terminal.

The proton-magnetic-resonance spectrum of bacilysin in D_2O showed a doublet at 8.41τ (J 8 Hz) attributable to the protons of the methyl group of the alanine residue. Signals at 5.83 and 5.9 τ could come from the proton attached to the α -carbon of this residue and a triplet at 5.68 τ (J 7 Hz) to the corresponding proton of the $-\text{CH}_2 \cdot \text{CH} \cdot \text{CO}_2\text{H}$ portion of the tyrosine-yielding residue. A pair of doublets at 6.30 and 6.61 τ (J 3 Hz) could be assigned to two epoxide protons. The value for the coupling constant is similar to that found in known $\alpha\beta$ -epoxyketones (Bhacca & Williams, 1964).

Since the signals from the epoxide protons were doublets it appeared that these protons were only coupled with each other. The only structure consistent with this finding was one in which one epoxide proton was adjacent to the carbonyl group (C-1) and the other to the carbon (C-4) carrying the side chain of the cyclohexanone ring. If the dihedral angle between the single hydrogen at C-4 and that at the adjacent epoxide carbon were 90° a coupling constant of zero between these protons would be predicted (Tori, Komeno & Nakagawa, 1964; Bhacca & Williams, 1964). Molecular models indicated that structures (I) and (II) would meet this requirement.

Further evidence for the presence of an $\alpha\beta$ -epoxyketone group in bacilysin was obtained by reduction of the antibiotic with chromous chloride solution. After 20 min the u.v.-absorption spectrum of the reaction mixture showed λ_{max} 231 nm and the extinction corresponded to that of a product showing $\epsilon 7.55 \times 10^3$. This absorption spectrum could be attributed to an $\alpha\beta$ -unsaturated ketone. $\alpha\beta$ -

Fig. 1. Optical-rotatory-dispersion curve of bacilysin. For details see the Experimental section. $[\Phi] = [\alpha] \times \text{mol.wt./100}$.

Epoxyketones are known to be reduced rapidly by chromous chloride to the corresponding $\alpha\beta$ -unsaturated ketone, probably via a hydroxyketone intermediate (Cole & Julian, 1954). The product from bacilysin yielded alanine and an uncharacterized amino acid with R_{Tyr} 1.1 on paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.) when hydrolysed with 6M-hydrochloric acid at 105°C for 18 h.

The optical-rotatory-dispersion curve of bacilysin was relevant to a decision between structures (I) and (II). This curve (Fig. 1) gave a positive Cotton effect with a peak at 305 nm and a trough at 271 nm (α , +20.75), associated with the $n \rightarrow \pi^*$ transition of the keto group, and a second Cotton effect with a peak at 228 nm and a trough at 213 nm (α , +58.5), which could be attributed to the amide and the carboxyl group. The sign and magnitude of the Cotton effect for saturated ketones carrying alkyl and halogen substituents were related to the stereochemistry of the molecule by an Octant rule that used

Fig. 2. Application of the reversed Octant rule to bacilysin. The diagram shows a near-planar conformation of the epoxycyclohexanone ring of the antibiotic. Carbon atoms are numbered as in structure (I). O represents oxygen. + and - refer to positive and negative octants.

the symmetry planes of the orbitals involved in the $n \rightarrow \pi^*$ transition as a frame of reference (Moffitt, Woodward, Moscovitz, Klyne & Djerassi, 1961). Subsequently a reverse Octant rule was found to apply to $\alpha\beta$ -epoxyketones (Djerassi, Klyne, Novin, Ohloff & Klein, 1965). Exceptions were encountered and it was suggested that in these cases the contribution from the epoxide ring was outweighed by that from other parts of the molecule (Kuriyama, Tada, Sawa, Ito & Itoh, 1968). In the case of bacilysin the reverse Octant rule predicts (from the positive Cotton effect) that the epoxide ring will be in a positive Octant. A near-planar conformation of the epoxycyclohexanone ring of the antibiotic in which this is so is shown in Fig. 2, the planarity resulting largely from the presence of the epoxide grouping. The substituent at C-4 lies almost in the vertical plane dividing the Octant that passes through C-1 and C-4. Hence, according to the Octant rules, it should not contribute to the Cotton effect. On this basis it can be deduced that structure (I) rather than structure (II) is correct, and that (III) represents the structure of bacilysin.

Substance AA1 was indistinguishable on paper chromatography in butan-1-ol-acetic acid-water and propan-2-ol-water and on paper electrophoresis at pH 1.8 and 4.5 from a substance obtained (together with alanine) by hydrolysis of bacilysin with leucine aminopeptidase.

The mass spectrum of a derivative of substance AA1 obtained by trifluoroacetylation and methylation with methanolic hydrochloric acid showed a peak at m/e 309, corresponding to the molecular ion of the methyl ester of the *N*-trifluoroacetyl derivative of an amino acid with mass 199. Much of the spectrum was similar to that of the methyl ester of the *N*-trifluoroacetyl derivative of bacilysin. A

peak at m/e 323 could be assigned to the molecular ion of a compound formed by addition of methanol across an epoxide ring and subsequent dehydration to give a vinyl ether, as could the peak at $M+14$ in the mass spectrum of bacilysin.

The proton-magnetic-resonance spectrum of substance AA1 in D_2O contained two doublets at 6.6 and 6.27 τ (J 4 Hz), corresponding (as with bacilysin) to two epoxide protons coupled only to each other. It also contained a triplet at 6.12 τ (J 6 Hz), which could be assigned to a proton on the

α -carbon of a $-CH_2-CH-CO_2^-$ grouping.

The optical-rotatory-dispersion curve of substance AA1 showed a positive Cotton effect with a peak at 311 nm and a trough at 260 nm (α , +23.8), indicating the presence of a carbonyl group, and a peak at 220 nm, which could be due to a carboxyl group of an L-amino acid (Fig. 3) (Jennings, Klyne & Scopes, 1965).

These results led to the conclusion that substance AA1 has the structure (IV) and was identical with the C-terminal amino acid of bacilysin.

Only alanine and tyrosine (mole ratio 1:0.79) were detected in an acid hydrolysate of bacilysin (6M-hydrochloric acid at 110°C for 17 h) when the latter was subjected to paper chromatography in butan-1-ol-acetic acid-water and to electrophoresis on paper at pH 4.5 and 1.8. In a similar experiment only tyrosine was detected in a hydrolysate of substance AA1. Nevertheless, when the hydrolysates were run in an amino acid analyser a further substance was detected that emerged from the column between 3,4-dihydroxyphenylalanine and tyrosine. This substance was indistinguishable from authentic *m*-tyrosine (*m*-hydroxyphenylalanine) when the two substances were examined together in the amino acid analyser. It was resolved from tyrosine when chromatographed on paper in butan-1-ol-aq. ammonia-dioxan (see the Experimental section), the R_{A1a} values of tyrosine and *m*-tyrosine in this system being 0.64 and 0.76 respectively. *m*-Tyrosine gave a bright yellow spot on paper with the diazotized sulphanilic acid

Fig. 3. Optical-rotatory-dispersion curve of compound AA1. For details see the Experimental section.

reagent (Ames & Mitchell, 1952), whereas tyrosine gave a tomato-red colour. Analysis in the amino acid analyser indicated that the tyrosine/*m*-tyrosine mole ratio was 4.5:1 in the hydrolysate of bacilysin and 10:1 in the material obtained by treating substances AA1 with 6*M*-hydrochloric acid under the same conditions. This difference is presumably due to the influence of the alanyl residue on electronic displacements in the ring. The aromatization of other $\alpha\beta$ -epoxyketones under acidic conditions has been reported by a number of workers (Wolff & Karash, 1959; Kaufmann, 1966; Vanderwalle, Seghal & Sipido, 1968), but no information about the detailed mechanism of the process appears to be available.

A proposed scheme for the formation of some of the more abundant molecular ions in the mass spectra of the compounds obtained by methylation of the *N*-trifluoroacetyl derivatives of bacilysin and substance AA1 is given in Scheme 1. A number of ions from both compounds can be accounted

for on the assumption that they are derived from vinyl ethers (structure V from substance AA1 and structure VI from bacilysin) formed after attack of methanol on the epoxide ring. These compounds appear to aromatize readily, either by electron impact or a thermal process.

Substance AA1 (2,3-epoxy-4-oxohexahydro-phenylalanine) showed less than 1.0 unit/mg when assayed against *Staphylococcus aureus* (N.C.T.C. 6571), a finding in agreement with the observation of Rogers *et al.* (1965) that bacilysin lost its activity when hydrolysed with leucine aminopeptidase. However, two transformation products of bacilysin with significant activity were encountered in the present work. Electrophoresis on paper at pH 4.5 and subsequent bioautography of a purified preparation of bacilysin that had been stored for several months revealed the presence of two minor active compounds in addition to bacilysin itself. One of these compounds migrated towards the cathode and showed $R_{\text{bacilysin}}$ 0.37 on paper chromatography in butan-1-ol-acetic acid-water, and the other migrated towards the anode and showed the same R_f value as bacilysin in butan-1-ol-acetic acid-water. Two experiments showed that the former basic compound was formed spontaneously from neutral bacilysin in aqueous solution. It was detected after electrophoresis, at pH 4.5, of bacilysin that had been eluted from paper after previous electrophoresis under these conditions; it increased in amount when a solution of bacilysin in 0.1 *M*-acetic acid was kept at room temperature for 72 h. On hydrolysis with 6*M*-hydrochloric acid at 110°C it yielded alanine but no tyrosine. The lactone structure (VII) has been tentatively considered for this compound. The nature of the acidic compound, which showed only traces of activity on bioautography, has not been ascertained.

The zones of inhibition of *Staph. aureus* produced by the basic compound contained colonies of resistant mutants. Since these resistant organisms were also cross-resistant to bacilysin it appears that the two substances may resemble each other in their mode of action. Several reactions that result in fission of the epoxide ring in bacilysin are known to yield inactive products (Rogers *et al.* 1965). However, if structure VII were correct an intact epoxide would not necessarily be essential for a bacilysin type of antibacterial activity.

Compound P2, which was isolated, together with bacilysin and substance AA1, from the culture fluid of *B. subtilis* A14 (Walker & Abraham, 1970) yielded only alanine when hydrolysed with 6*M*-hydrochloric acid at 110°C for 17 h or with leucine aminopeptidase. The mass spectrum of the methyl ester of its *N*-trifluoroacetyl derivative gave a molecular ion of 270 and ions with *m/e* 239 and 211, corresponding to the loss of $\text{O}\cdot\text{CH}_3$ and $\text{CO}\cdot\text{O}\cdot\text{CH}_3$,

Scheme 1. Proposed mechanism of the formation of some abundant positive ions detected in the mass spectrum of a preparation of the methyl ester of the *N*-trifluoroacetyl derivative of compound AA1 ($R = CF_3 \cdot CO$) and bacilysin [$R = CF_3 \cdot CO \cdot NH \cdot CH(CH_3) \cdot CO$].

respectively. An abundant ion at m/e 44, corre-

sponding to CH_3NH_2^+ , indicated the presence of a C -terminal alanyl residue (Stenhagen, 1961) and

ions with m/e 140 ($\text{CH}_3\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot$) and 141 indicated an N -terminal alanine residue. In these and other respects the spectrum closely resembled that of the methyl ester of the N -trifluoroacetyl derivative of alanylalanine (Stenhagen, 1961). Substance P2 was indistinguishable from synthetic DL-alanyl-DL-alanine on paper electrophoresis at pH1.8 and pH4.5 and on paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.) and in propan-2-ol-water (7:3, v/v). In view of its rapid hydrolysis by leucine aminopeptidase it can thus be concluded that compound P2 is L-alanyl-L-alanine.

Compound P3 (Walker & Abraham, 1970) also yielded alanine on hydrolysis with 6M-hydrochloric acid at 110°C and with leucine aminopeptidase, although with this substance the hydrolysates were contaminated with traces of other amino acids. The finding that substance P3 migrated less far towards the anode than substance P2 or bacilysin on electrophoresis at pH1.8 suggested that it was a peptide with more than two amino acid residues. It may be a poly-L-alanine, but no further decisive information has been obtained about its structure.

Alanine acts as a precursor of the N -terminal L-alanine residue of bacilysin when added to cultures of *B. subtilis* A14 (Roscoe & Abraham, 1966) and it is of note that all three of the peptides isolated from the culture fluid contain alanine as a major constituent. Whether substance AA1, the C -terminal amino acid of bacilysin, is itself a precursor of the antibiotic remains to be determined, but no evidence was obtained that this amino acid was produced by enzymic hydrolysis of bacilysin when the latter was added to culture supernatants of the organism. Roscoe & Abraham (1966) showed that ^{14}C from [^{14}C]shikimic acid was incorporated into bacilysin by *B. subtilis*. The structure of bacilysin (III) suggests that a possible precursor would be

prephenic acid (VIII). Epoxidation of one double bond in (VIII), reduction of the second and oxidation of the alcoholic group to a keto group could yield the epoxycyclohexanone ring of the antibiotic. In this case an essential step in the biosynthesis of bacilysin would involve a diversion from the pathway to the aromatic amino acids near its terminal stage.

EXPERIMENTAL

Chemicals. D_2O was from Imperial Chemical Industries Ltd., London, E.C.4, U.K., and was of isotopic purity 99.7 atoms % D. *NO*-Bis(trimethylsilyl)acetamide and DL-alanyl-DL-alanine were from the Sigma Chemical Co., St Louis, Mo., U.S.A., and leucine aminopeptidase was from the Boehringer Corp. (London) Ltd., London W.5, U.K. Samples of *o*-tyrosine, *m*-tyrosine and 3,4-dihydroxyphenylalanine were given by Dr H. Blaschko.

Chromatography and electrophoresis. Paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.) and propan-2-ol-water (7:3, v/v) on Whatman no. 1 paper, paper electrophoresis at pH1.8 and pH4.5 at 60V/cm, elution from paper and bioautography were as described by Walker & Abraham (1970). Paper chromatography was also carried out in the upper layer of butan-1-ol-dioxan-2M- NH_3 (4:1:5, by vol.) (Brown & Jackson, 1954). Quantitative amino acid analyses were carried out in an amino acid analyser (from Evans Electroselenium Ltd., Halstead, Essex, U.K.) by the procedure of Spackman, Stein & Moore (1958).

Mass spectra. These were kindly recorded by Dr R. T. Aplin of the Dyson Perrins Laboratory, Oxford, U.K., with an AEI M59 Mass Spectrometer operating at 70eV.

Proton-magnetic-resonance spectra. Samples (about 0.1mmol) of substances whose spectra were to be determined were dissolved in 1ml of D_2O and the solutions were freeze-dried. The samples were then redissolved in D_2O and their spectra were measured by Mrs R. E. Richards of the Dyson Perrins Laboratory in a Perkin-Elmer R14 (100MHz) spectrometer, with 3-(trimethylsilyl)propanesulphonate as an internal standard.

Optical-rotatory-dispersion curves. These were kindly measured by Dr P. M. Scopes and Professor W. Klyne, Westfield College, London N.W.3, U.K. The samples (about 3mg) were dissolved in water to give a concentration of about 2mg/ml and the measurements were made at room temperature with a 0.1cm cell in a Bellingham and Stanley/Bendix-Ericsson automatic recording spectropolarimeter.

Reduction of bacilysin with chromous chloride. A light-blue solution of CrCl_2 was prepared as described by Rosenkranz, Mancera, Gatica & Djerassi (1950) and was kept under CO_2 until required.

The CrCl_2 solution (75 μ l) was added to 1 ml of a stirred aqueous solution of bacilysin (1.04 mg, 260 units/mg) under a stream of CO_2 . Samples were removed at intervals and their u.v.-absorption spectra were measured (after suitable dilution) against an appropriate blank. The value of E_{230} reached a maximum within 20 min.

Methyl esters of N-trifluoroacetyl derivatives of peptides and amino acids. These compounds were prepared on a small scale by a modification of the method of Senn, Venkataraghavan & McLafferty (1966). The substance (0.1–0.6 μ mol) was first esterified with 40 $\pm$ 10 μ l of 2–2.5 M-HCl in methanol. The reaction was carried out in a small flask made by blowing a bulb of 6 mm diam. at the end of a 2 mm-diam. capillary tube, the substance being added from a micro-spatula and the methanolic HCl from a micro-pipette. The top of the capillary tube was sealed and the reaction mixture was kept at room temperature for 16 h. The capillary was then opened and the solvent removed *in vacuo*. The residue was dissolved in 40 $\pm$ 10 μ l of a mixture of trifluoroacetic acid and trifluoroacetic anhydride (1:2, v/v) in the same reaction vessel. The latter was sealed and left at room temperature for 3 h. The tube was then opened and the volatile reagents were removed *in vacuo*.

Trimethylsilyl derivative of bacilysin. To a solution of bacilysin (0.53 mg, 260 units/mg) in dry dimethylformamide (500 μ l) was added 50 μ l of *NO*-bis(trimethylsilyl)-acetamide (Klebe, Finkbeiner & White, 1966). The reaction tube was stoppered and kept in a desiccator overnight. The solvent and excess of reagent were removed in a high vacuum, leaving a white solid that was soluble in benzene.

Hydrolysis with leucine aminopeptidase. This was carried out in a buffer containing ammonium acetate (0.1 M) and MnCl_2 (2.5 mM). To a solution of bacilysin (2.0 mg, 260 units/mg) or substance P2 (1.2 mg) in 0.25 ml of buffer was added 10 μ l of a solution of leucine aminopeptidase (5 mg/ml). The mixture was kept at 37°C, and samples (25 μ l) were removed at 1, 2 and 4 h and applied to paper for chromatography in butan-1-ol-acetic acid-water. The appearance of the chromatograms after spraying with ninhydrin indicated that hydrolysis of both substances was virtually complete in 1 h.

Effect of culture supernatants from *B. subtilis* A14 on bacilysin. Supernatant (49 ml) from a culture of *B. subtilis* A14 containing 30 units of bacilysin/ml was sterilized by filtration through a Millipore filter (no. HAWG 025) and divided into four equal parts. Two of the latter were boiled for 10 min (without loss of bacilysin activity) to destroy enzymic activity. Partially purified bacilysin (20 units/mg) was added to both of the boiled and to one unboiled sample to give a final antibacterial activity of 55 units/ml, and the pH of the solutions was adjusted to 7.0. A further solution of bacilysin (12 ml, 55 units/ml) was made in 0.1 M-sodium-potassium phosphate buffer, pH 7.0. All four solutions were shaken in 1-tubes (as described by Roscoe & Abraham, 1966) in a water bath at 33–34°C and samples were removed at intervals for antibacterial assay. After 10 h the antibacterial activities of all the samples had fallen to about 90% of their original values. The losses in activities of the different solutions did not differ significantly from each other.

Note added. After the work described in this paper had been done we were informed by Dr E. H. Flynn of the discovery and study in the Lilly Research Laboratories of a substance named Anticapsin which is produced by a strain of *Streptomyces griseoplanus* and inhibits the synthesis of the capsule of *Streptococcus pyogenes*. Entirely independently of the work on bacilysin, Neuss, Molloy, Shah & DeLaHiguera (1970) have obtained evidence for a structure for anticapsin which is identical with that proposed for substance AA1 (IV). Dr Neuss kindly provided us with a sample of anticapsin. The optical-rotatory-dispersion curve of this substance was similar in sign and shape to that of substance AA1. The two substances behaved in an identical manner when subjected to electrophoresis on paper at pH 1.8 and chromatography in butan-1-ol-acetic acid-water in a perpendicular direction.

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REFERENCES

- Ames, B. N. & Mitchell, H. K. (1952). *J. Am. chem. Soc.* **74**, 252.
Bhacca, N. S. & Williams, D. H. (1964). *Applications of n.m.r. Spectroscopy in Organic Chemistry*, p. 99. San Francisco, London and Amsterdam: Holden-Day Inc.
Biemann, K. (1962). *Mass Spectrometry: Organic Chemical Applications*, p. 172. New York, San Francisco, Toronto and London: McGraw-Hill Inc.
Brown, F. & Jackson, H. (1954). *Biochem. J.* **56**, 399.
Cole, W. & Julian, P. L. (1954). *J. org. Chem.* **19**, 131.
Djerassi, C., Klyne, W., Novin, T., Ohloff, G. & Klein, E. (1965). *Tetrahedron*, **21**, 163.
Jennings, J. P., Klyne, W. & Scopes, P. M. (1965). *J. chem. Soc.* p. 294.
Kaufmann, S. (1966). *J. org. Chem.* **31**, 2395.
Klebe, J. F., Finkbeiner, H. & White, D. M. (1966). *J. Am. chem. Soc.* **88**, 3390.
Kuriyama, K., Tada, H., Sawa, Y. K., Ito, S. & Itoh, I. (1968). *Tetrahedron Lett.* no. 21, p. 2539.
Moffitt, W., Woodward, R. B., Moscowitz, A., Klyne, W. & Djerassi, C. (1961). *J. Am. chem. Soc.* **83**, 4013.
Neuss, N., Molloy, B. B., Shah, R. & DeLaHiguera, N. (1970). *Biochem. J.* **118**, 571.
Rogers, H. J., Lomakina, N. & Abraham, E. P. (1965). *Biochem. J.* **97**, 579.
Roscoe, J. & Abraham, E. P. (1966). *Biochem. J.* **99**, 793.
Rosenkranz, G., Mancera, O., Gatica, J. & Djerassi, C. (1950). *J. Am. chem. Soc.* **72**, 4077.

- Senn, M., Venkataraghavan, R. & McLafferty, F. W. (1966). *J. Am. chem. Soc.* **88**, 5593.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Stenhagen, E. (1961). *Z. analyt. Chem.* **181**, 462.
- Tori, K., Komeno, T. & Nakagawa, T. (1964). *J. org. Chem.* **29**, 1136.
- Vanderwalle, M., Seghal, K. L. & Sipido, V. (1968). *Bull. Soc. chim. Belg.* **77**, 611.
- Walker, J. E. & Abraham, E. P. (1970). *Biochem. J.* **118**, 557.
- Weygand, F., Prox, A., Fessel, H. H. & Sun, K. K. (1965). *Z. Naturf.* **20b**, 1169.
- Wolff, M. E. & Karash, C. B. (1959). *J. org. Chem.* **24**, 1612.