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THE NUCLEAR MAGNETIC RESONANCE AND MASS SPECTRA OF DERIVATIVES OF CYCLOSERINE

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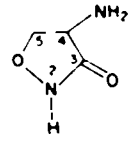
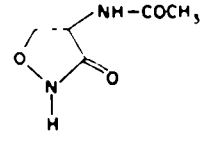
Abstract—On the basis of NMR spectral data, it is shown that acetylation of the 3-isoxazolidone ring of N-acetylcycloserine occurs on nitrogen *whereas* sulfonylation occurs on the carbonyl oxygen. The change in conformation (dihedral angles) resulting from introduction of a double bond into the heterocyclic ring is reflected in a consistent displacement of the parameters of the ABX system. Variation in the site of acylation is considered explicable in terms of kinetic vs. thermodynamic control. The mass spectra of various derivatives of cycloserine are shown to be compatible with the assigned structures. Some unusual modes of fragmentation of the 4-amino-3-isoxazolidone system are discussed.

SINCE its isolation and characterization in 1955,[2,3] cycloserine (I), which is alternatively known as oxamycin and by its systematic name, D-4-amino-3-isoxazolidone, has been the subject of continued study directed at an understanding of its biological activity and the chemistry of the isoxazolidone system. Cycloserine owes its biological activity at least in part to its ability to inhibit pyridoxal-dependent reactions[4] such as enzymatic transamination, decarboxylation and racemization. The manner in which this inhibition is achieved has been thought[5,6] to involve selective reaction of the cycloserine with pyridoxal. Stammer and McKinney[7] have recently characterized, as a model for this reaction, the Schiff base formed by condensation of cycloserine with a substituted benzaldehyde. Consideration has also been given to the possible role of the N-acylaminoxy function in certain enzyme-catalysed hydrolyses.[8] To judge from these earlier studies with cycloserine, there is reason to suppose that the more definitive nucleophile, N-acetylcycloserine (II) might assist in acyl transfer reactions similar to those catalysed by imidazole,[9] as defined schematically below:



I



II

[1] Visiting Fellow, U.S. Public Health Service, 1962–1965.

[2] F. A. Kuehl, Jr., F. J. Wolf, N. R. Trenner, R. L. Peck, E. Howe, B. D. Hunnewell, G. Downing, E. Newstead, R. L. Buhs, I. Putter, R. Ormond, J. E. Lyons, L. Chaiet and K. Folkers, *J. Amer. Chem. Soc.* 77, 2344 (1955).

[3] P. H. Hidy, E. B. Hodge, V. V. Young, R. L. Harned, G. A. Brewer, W. F. Phillips, W. F. Runge, H. E. Stavely, A. Pohland, H. Boaz and H. R. Sullivan, *J. Amer. Chem. Soc.* 77, 2345 (1955).

[4] J. L. Strominger, *Physiol. Rev.* 40, 87 (1960).

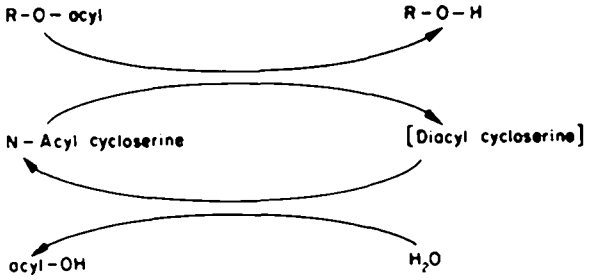
[5] J. Michalsky, J. Opichal and J. Ctvrtnik, *Monatsh.* 93, 618 (1962).

[6] N. K. Kochetkov, *Osterr. Chem. Zeit.* 62, 276 (1961).

[7] C. H. Stammer and J. D. McKinney, *J. Org. Chem.* 30, 3436 (1965).

[8] T. Viswanatha, *Proc. Nat. Acad. Sci., U.S.* 50, 967 (1963).

[9] L. Mandell, J. W. Moncrieff and J. H. Goldstein, *Tetrahedron* 19, 2025 (1963), and Refs. cited therein.

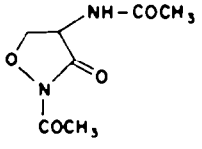


The effective result of such a series of reactions is hydrolysis of an ester to acid and alcohol by water, the diacyl cycloserine serving as a reactive intermediate. Among the prerequisites for such a scheme to be successful, this "diacyl cycloserine" must be labile at the pH of the system and the function of N-acyl cycloserine in a catalytic role should be therefore demonstrable.

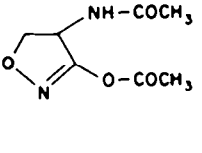
In view of the potential for tautomerism in the 3-isoxazolidone ring system,[3] the acylation of N-acetylcycloserine (II) may occur either on nitrogen or on carbonyl oxygen. This paper deals with the site of acylation and the structure of the resulting products.

As has been noted previously,[2,3,10] the primary amino group of cycloserine can be smoothly acetylated under a variety of conditions, typically, in methanol with acetic anhydride at room temperature,[10] to give N-acetylcycloserine, II. This is a normal acetylation of a primary amine and as such is reversible only by drastic acid or alkaline hydrolysis. Subsequent treatment of II with acetic anhydride in pyridine for 0·5 hr at room temperature leads to the formation, in 70–75% yield, of a crystalline product which was characterized by its NMR spectrum, mass spectrum and elementary analysis as a diacetyl cycloserine, $C_7H_{10}N_2O_4$. The product, m.p. 121–122°, was considered identical to that described in earlier reports.[3,11] This material is hydrolysed rapidly in aqueous solution at pH 7·0 and thus fulfills one of the requirements adduced above, that is, lability at essentially neutral pH.

There can be written, *a priori*, two structures (III and IV) which are consistent with the genesis, the lability and the molecular formula of diacetyl cycloserine.[12] The lability of III, *at neutral* pH, is to be anticipated on the basis of its being a



III



IV

diacylamine with an additional electron-withdrawing group adjacent to nitrogen. A suitable comparison may be made with N-acetylsuccinimide, which hydrolyses spontaneously at pH 6–7.[13] In the alternative formulation (IV), diacetyl cycloserine

[10] F. A. Kuehl, U.S. Pat. 2,845,432. *Chem. Abstr.* **52**, 20198d (1958).

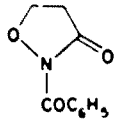
[11] I. Mitsui and S. Imaizumi, *J. Chem. Soc., Japan* **78**, 812 (1957).

[12] Additional possibilities in which the side-chain acetamido function becomes further acylated are excluded by the appearance of the side-chain NH in NMR spectra (*vide infra*).

[13] H. K. Hall, Jr., M. K. Brandt and R. M. Mason, *J. Amer. Chem. Soc.* **80**, 6420 (1958).

would be expected to be even more labile, being an ester of the thermodynamically unfavorable member of an amide–iminol equilibrium.[14]

Mitsui and Imaizumi[11] assigned to diacetyl cycloserine structure IV as a consequence of its conversion to serine aldehyde and ammonia by catalytic hydrogenation followed by hydrolysis. Hidy *et al.*[3] reported the physical constants for the same substance but advanced no structure for it. Benzoylation of either the silver salt or the potassium salt of *des*-aminocycloserine (3-isoxazolidone) is reported to give a single benzoyl derivative[15] which was assigned the N-benzoyl structure (V) on the basis of its UV and IR spectra. The uncertainties in structural assignment which arise in the cycloserine case serve merely to supplement those which have existed since the formulation of the amide-iminol concept.[16] Whether the alkylation of an amide has occurred on oxygen or nitrogen can often be determined by classical chemical techniques;[3] however, the site of acylation, as well as the predominant



V

form of the parent compound, must usually be determined on the basis of physical properties. When one of the tautomers includes a heteroaromatic ring in its structure, the problem is often solved by means of UV spectroscopy.[17] Such a technique is far from sufficient for simple amides which lack significant spectral characteristics and other physical methods must be utilized.

We have studied diacetyl cycloserine by NMR and mass spectrometry. On the basis of its NMR spectrum and by a comparison with other compounds of less equivocal structure, we consider diacetyl cycloserine to have structure III and to be therefore, N,2-diacetylcycloserine.

NMR spectra

The NMR spectrum of diacetyl cycloserine in pyridine-d_5 is shown in Fig. 1.[18] The singlet at 125·3 c/s is the signal from the acetyl group of the acetamido side chain, and the other singlet at 146·2 c/s is the signal from the second acetyl group. In N-acetylcycloserine, the lower field singlet is absent.

The group of fifteen peaks between 255 and 345 c/s is the signal from three protons, H_A, H_B and H_X in structure VI. These three protons give essentially a

[14] C. L. Stevens and M. E. Munk, *J. Amer. Chem. Soc.* **80**, 4065 (1958); H. G. Khorana, *Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest* p. 126. John Wiley, New York, N.Y. (1961).

[15] N. K. Kochetkov, R. M. Khomutov, M. Ya. Karpeiskii, E. I. Budovski and V. I. Erashko, *Zhur. Obschei Khim.* **29**, 3417 (1959).

[16] G. W. Wheland, *Advanced Organic Chemistry* pp. 617–623. John Wiley, New York, N.Y. (1949); C. A. Grob and B. Fischer, *Helv. Chim. Acta*, **38**, 1794 (1955).

[17] A. R. Katritzky and J. M. Lagowski, in *Advances in Heterocyclic Chemistry*, Vol. I (Edited by A. R. Katritzky) pp. 312, 341. Academic Press, New York, N.Y. (1963).

[18] All NMR spectra were measured on 0·5M solutions in pyridine or pyridine-d_5 with a Varian Associates A-60 spectrometer fitted with a variable temperature probe operating at 37 ± 2°. Frequencies are expressed relative to an internal standard of tetramethylsilane and are reproducible to at least 0·1 c/s.

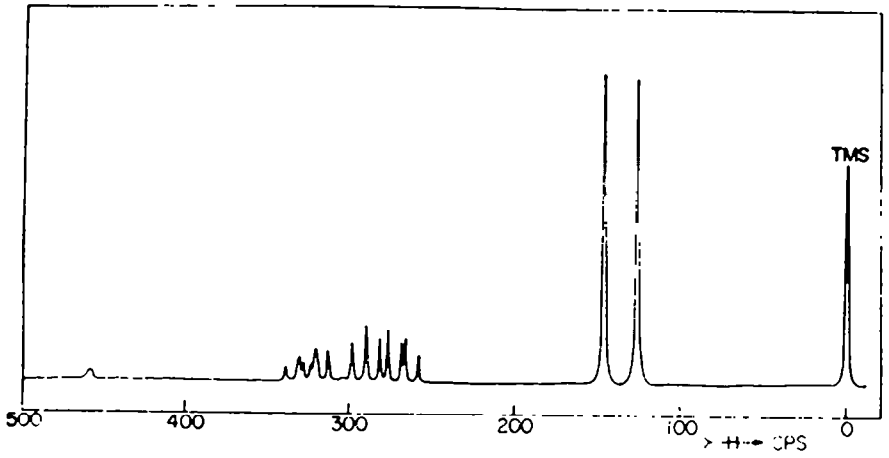
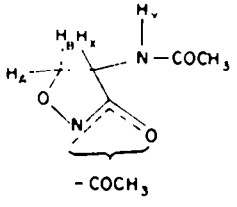


FIG. 1. The NMR spectrum of diacetyl cycloserine in pyridine-d_5.



VI

classical ABX spectrum, whose X portion is additionally complicated by the perturbation of the X signal by H_Y. Thus the AB portion of the spectrum consists, as is usual, of two quartets which, in this case, overlap in such a way as to reduce the total number of peaks from eight to seven.

The X portion, theoretically a sextet, fails to show the two outer combination lines[19] and the resulting quartet is so perturbed by the neighboring proton on nitrogen (H_Y) as to appear as an octet. This fourth proton, H_Y, gives a broad signal at about 445 c/s which is obscured if nondeuterated pyridine is used as solvent.

The spectrum may therefore be analysed as an ABX spectrum by the method of Pople, Schneider and Bernstein[20] with reference to Fig. 2.

The interval J_{AB} is repeated four times as the separation between lines 8 and 6, 4 and 2, 7 and 5, and 3 and 1. Thus a mean value can be derived and is found to be

$$J_{AB} = \pm 8{\cdot}1 \text{ c/s}$$

[19] These combination lines are prohibited for an AMX system but not for an ABX system (cf. J. D. Roberts, *An Introduction to the Analysis of Spin-Spin Splitting in Nuclear Magnetic Resonance* p. 71 *et seq.* W. A. Benjamin Inc. (1962). The spectrum under consideration here may well be supposed to be intermediate between these two extremes, the result being the non-appearance of the weak combination lines above the background. This was confirmed when it was found that recalculation of the spectrum from the extracted parameters (*vide infra*) gave the observed octet for the X portion unless coupling to Y was ignored, in which case the X portion appeared as a quartet.

[20] J. A. Pople, W. G. Schneider and H. J. Bernstein, *Can. J. Chem.* **35**, 65 (1957).

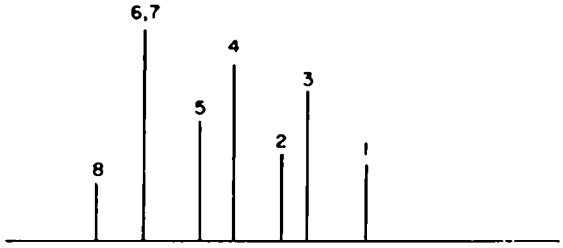


FIG. 2. AB Portions of the ABX spectrum in Fig. 1. Lines 2, 4, 6 and 8 are A lines and lines 1, 3, 5 and 7 are lines from the B proton.

The parameter D_+ is half the separation between lines 8 and 4 or 6 and 2 and thus

$$D_+ = 10{\cdot}8 \text{ c/s.}$$

Similarly, D_- is half the separation between lines 7 and 3 or 5 and 1 and so

$$D_- = 12{\cdot}3 \text{ c/s.}$$

Now
$$D_+ \cos 2\phi_+ = \tfrac{1}{2}(\nu_A - \nu_B) + \tfrac{1}{4}(J_{AX} - J_{BX})$$
$$D_+ \sin 2\phi_+ = \tfrac{1}{2}J_{AB}$$
$$D_- \cos 2\phi_- = \tfrac{1}{2}(\nu_A - \nu_B) - \tfrac{1}{4}(J_{AX} - J_{BX})$$
$$D_- \sin 2\phi_- = \tfrac{1}{2}J_{AB}$$

Elimination of ϕ_+ and ϕ_- from these equations gives

$$D_+^2 = (x + y)^2 + \tfrac{1}{4}J_{AB}^2$$

and

$$D_-^2 = (x - y)^2 + \tfrac{1}{4}J_{AB}^2$$

where $x = \tfrac{1}{2}(\nu_A - \nu_B)$ and $y = \tfrac{1}{4}(J_{AX} - J_{BX})$.
Substitution for D_+, D_- and J_{AB} gives

$$117{\cdot}3 = (x + y)^2 + 16{\cdot}6$$
$$152{\cdot}0 = (x - y)^2 + 16{\cdot}6$$

thus
$$(x + y) = 10{\cdot}0$$

and
$$(x - y) = 11{\cdot}6$$

Resubstitution for x and y gives

$$(\nu_A - \nu_B) = 21{\cdot}6 \text{ c/s and } \tfrac{1}{4}(J_{AX} - J_{BX}) = -0{\cdot}8$$

i.e. $(J_{AX} - J_{BX}) = -3{\cdot}2$
The separation between the centers of the A quartet and the B quartet is $\tfrac{1}{2}(J_{AX} + J_{BX})$

thus
$$(J_{AX} + J_{BX}) = 19{\cdot}5 \text{ c/s}$$

hence
$$J_{AX} = 8{\cdot}1 \text{ c/s and } J_{BX} = 11{\cdot}4 \text{ c/s.}$$

The sum of the frequencies of lines 2, 3, 6 and 7 is equal to $4\nu_{AB}$

where $\nu_{AB} = \frac{1}{2}(\nu_A + \nu_B)$

so $(\nu_A + \nu_B) = 556{\cdot}6$

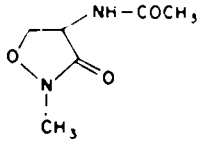
but $(\nu_A - \nu_B) = 21{\cdot}6$

hence $\nu_A = 289{\cdot}1$ c/s and $\nu_B = 267{\cdot}5$ c/s.

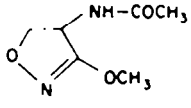
The X portion of the spectrum must be symmetrical about the frequency ν_X which is therefore 325·6 c/s and the overall width of the X octet will be, to a first approximation, $J_{AB} + J_{BX} + J_{XY}$. This leads to a value for J_{XY} of 7·3 c/s which is quite consistent with the figure of 7·5 c/s found by Tori and Kuriyama[21] between the NH proton and the 6α-proton in 6β-acetamidopregn-4-ene-3,2—dione.

The seven parameters thus derived were recombined by a Honeywell 800 digital computer[22] and found to give a theoretical spectrum which corresponded in every detail with the observed spectrum.

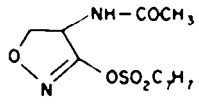
In this way, NMR spectra were recorded, analysed and calculated for N-acetylcycloserine (anion), N-acetyl-2-methylcycloserine (VII),[3] N-acetyl-0-methylcycloserine[3] (VIII) and the previously unknown *p*-toluenesulfonate ester of N-acetylcycloserine. This *p*-toluenesulfonate was tentatively considered to be the 0-sulfonate (IX) since it could be hydrolysed by dilute alkali to give *p*-toluenesulfonic acid, detectable by high voltage electrophoresis.[23] Instances of amides being esterified at oxygen by *p*-toluenesulfonyl chloride are uncommon but not unknown.[24]



VII



VIII



IX

Inspection of the seven parameters derived for each compound and recorded in Table 1, shows that a clear distinction can be made between N-acetylcycloserine and N-acetyl-2-methylcycloserine on the one hand, and N-acetyl-0-methylcycloserine (VIII) and N-acetyl-0-*p*-toluenesulfonylcycloserine on the other and that the parameters for diacetyl cycloserine place it in the former group, thus allowing the assignment of structure III to this compound.

When the double bond is introduced into the ring, J_{AB} increases by about 1 c/s and this may be due to a decrease in the H_A–C_6–H_B angle although as has been

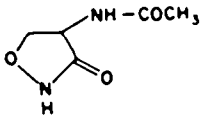
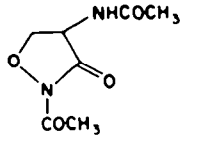
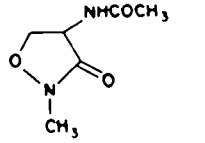
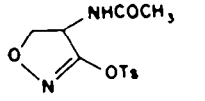
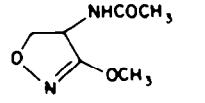
[21] K. Tori and K. Kuriyama, *Chem. and Ind.* 1525 (1963).

[22] The chemical shift of H_Y does not affect this spectrum and this quantity was therefore arbitrarily set at 450·0 c/s. Calculation of spectra was carried out using a modified Freqint III program. We are very grateful to Dr. Aksel Bothner-By of the Mellon Institute for making this program available to us.

[23] It is probable that the reaction of an N-*p*-toluenesulfonyl derivative with alkali would result only in ring opening. It has been shown that N-methanesulfonylpyrrolidone reacts with alkali exclusively at the ring carbonyl (H. K. Hall, Jr., M. K. Brandt and R. M. Mason, *J. Amer. Chem. Soc.* **80**, 6420 (1958)).

[24] H. Plieninger, H. Bauer, A. R. Katritzky and U. Lerch, *Liebig's Ann.* **654**, 165 (1962).

TABLE 1. COUPLING CONSTANTS AND CHEMICAL SHIFTS DERIVED FROM THE NMR SPECTRA OF CYCLOSERINE DERIVATIVES (IN c/s).

Compound	J_{AB}	J_{AX}	J_{BX}	J_{XY}	ν_A	ν_B	ν_X	—$COCH_3$	Other
	8·6	8·5	9·9	6·1	293·1	259·8	328·7	128·2	—
	8·1	8·1	11·3	7·3	289·1	267·5	325·6	125·3	146·2
	8·5	8·4	9·7	7·0	283·1	249·6	316·0	126·4	186·7
	9·8	10·1	5·9	8·4	283·8	268·3	359·2	123·0	131·5
	9·4	9·3	6·4	7·8	280·2	260·1	341·2	126·4	226·5

pointed out recently,[25] care should be exercised in the application of this inverse relationship. Introduction of the double bond into the ring also causes J_{AX} to increase while J_{BX} decreases and in view of Karplus' relationship between coupling constant and dihedral angle,[26] this would be expected if the dihedral angle between H_A and H_X increases with a concomitant decrease in the dihedral angle between H_B and H_X. The coupling constant J_{XY} seems not to be affected by the position of the double bond, which is reasonable if there is free rotation about the C_4–N bond.

The chemical shifts of H_A and H_B in all five compounds are of little help in structure assignment, but when the double bond is in the ring, H_X is "allylic" and as a result, its chemical shift is moved 25–50 c/s to lower field. This chemical shift alone provides support for the assignment of structure III to diacetyl cycloserine. In structure IV, one might reasonable expect the value of the chemical shift for H_X to fall between those observed in the presence of the strongly electron-releasing and electron-withdrawing substituents of VIII and IX, respectively.

The NMR spectrum (Table 1, particularly J_{AX} and J_{BX}) suggests that the heterocyclic ring of N-acetylcycloserine is devoid of olefinic character and that it resembles the N-methyl considerably more than the 0-methyl derivative.[27]

[25] H. M. Hutton and T. Schaefer, *Can. J. Chem.* **41**, 684 (1963).

[26] M. Karplus, *J. Amer. Chem. Soc.* **85**, 2870 (1963).

[27] It is assumed that N-acetylcycloserine (pK'_a 5·80[9]) exists in the undissociated form in pyridine (pK_a 5·17).

The IR and UV spectra of the same five compounds are recorded in Table 2 and 3, respectively. It is immediately evident that such data are of little value in making structural assignments in the cycloserine series. The fact that the side-chain amide band for N-acetylcycloserine (1645 cm^{-1}) is not significantly different in position from that of N-acetyl-2-methylcycloserine suggests that the intramolecularly hydrogen-bonded structure, X, and, accordingly, the iminol form of the lactam, do not contribute significantly to the structure of II in the solid state.

TABLE 2. IR ABSORPTION MAXIMA—CARBONYL REGION[a]

Compound	—$NHCOCH_3$	N—CO—C_4	N—C_3	N—$COCH_3$
N-Acetylcycloserine	1645 cm^{-1}	1710 cm^{-1}	—	—
N,2-Diacetylcycloserine	1667	1765	—	1700[b]
N-Acetyl-2-methylcycloserine	1640	1720	—	—
N-Acetyl-0-methylcycloserine	1660	—	1632	—
N-Acetyl-0-*p*-tosylcycloserine	1655	—	1638	—

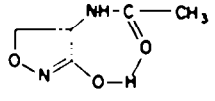
[a] All spectra were recorded in Nujol.

[b] The band at higher frequency is assigned to the more strained ring carbonyl (cf. H. K. Hall, Jr. and R. Zbinden, *J. Amer. Chem. Soc.* **80**, 6428 (1958)).

TABLE 3. UV ABSORPTION MAXIMA (ACETONITRILE)

Compound	λ_{max}
N-Acetylcycloserine	215 mμ[a]
N,2-Diacetylcycloserine	240
N-Acetyl-2-methylcycloserine	213[a]
N-Acetyl-0-methylcycloserine	210[a]
N-Acetyl-0-*p*-tosylcycloserine	228

[a] Inflection only



X

The reaction of N-acetylcycloserine with diazomethane leads to a mixture of the N-methyl and 0-methyl derivatives. The fact that a particular tautomer, which may contribute so little to the equilibrium mixture that it escapes detection by physical means, leads to a significant amount of product is a phenomenon with ample precedent,[17,28] and has no bearing on the elucidation of the structure of II. We consider the difference in site of acylation with *p*-toluenesulfonyl chloride and with acetic anhydride to represent, not a difference in reagent or mechanism, but one of ease of rearrangement. Under the conditions of the acylation reaction, IX is not likely to undergo deacylation and is, therefore, a product of kinetic control. We presume that acetic anhydride reacts with II, initially, to give the 0-acetyl derivative; the product can, in turn, acylate pyridine or acetate anion or IV, or even experience an

[28] L. A. Cohen and W. M. Jones, *J. Amer. Chem. Soc.* **85**, 3397 (1963); G. L. Schmir and L. A. Cohen, *Ibid.* **83**, 723 (1961).

intramolecular acyl migration via a four-center transition state, and the final product, III, is the result of thermodynamic control under conditions of reversibility.[29]

Mass spectra

Support for the structural assignments made on the basis of the NMR spectra is found in the mass spectra[30] of the derivatives II, III, and VII.

From the mass spectra of II and III, shown in Fig. 3, it can be immediately seen

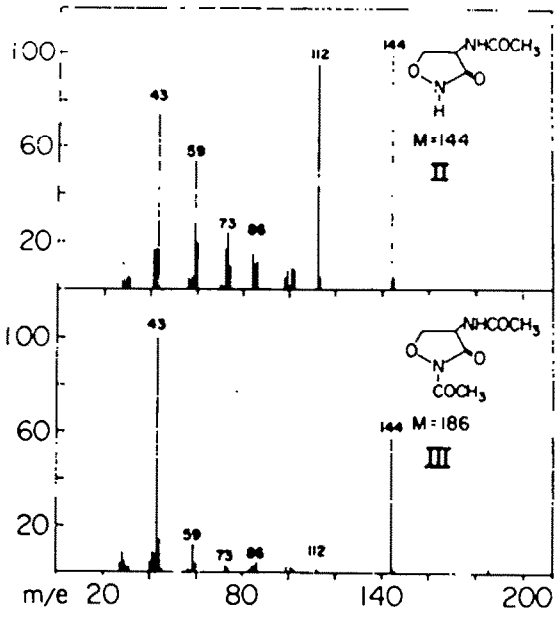


FIG. 3. Mass spectra of N-acetylcycloserine (II) and of N,2-diacetylcycloserine (III). The ordinate represents relative abundance.

that the normal N-acetyl fragmentations are occurring. Thus the molecular ion from II (m/e 144) loses ketene to give an ion of low abundance at m/e 102—a fragmentation with ample precedent[31,32] and, as an alternative process,[31,32] acetylium ion (CH_3CO^+) is lost from the molecular ion and carries most of the charge, thus accounting for the abundant ion at m/e 43 and the small peak at m/e 101.

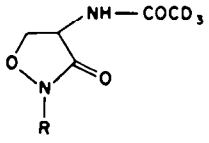
[29] The kinetic-thermodynamic system has been demonstrated experimentally for several ambident nucleophiles: see, for example, C. L. Stevens and M. E. Munk, *J. Amer. Chem. Soc.* **80**, 4065 (1958); R. E. Benson and T. L. Cairns, *Ibid.* **70**, 2115 (1948); W. P. Jencks, *Ibid.* **80**, 4581, 4585 (1958); D. Y. Curtin and L. L. Miller, *Tetrahedron Letters* 1869 (1965).

[30] All mass spectra were measured on an Associated Electrical Industries (U.K.) MS-9 double focussing mass spectrometer at 70 eV. In all cases, the sample was introduced directly into the electron beam. Accurate measurement of mass to charge ratio was carried out by comparison with a perfluorotributylamine standard. We are indebted to Drs. J. W. Daly and H. M. Fales of the National Institutes of Health for their assistance in these measurements.

[31] Z. Pelah, M. A. Kielczewski, J. M. Wilson, M. Ohashi, H. Budzikiewicz and C. Djerassi, *J. Amer. Chem. Soc.* **85**, 2470 (1963).

[32] A. M. Duffield and C. Djerassi, *J. Amer. Chem. Soc.* **87**, 4554 (1965).

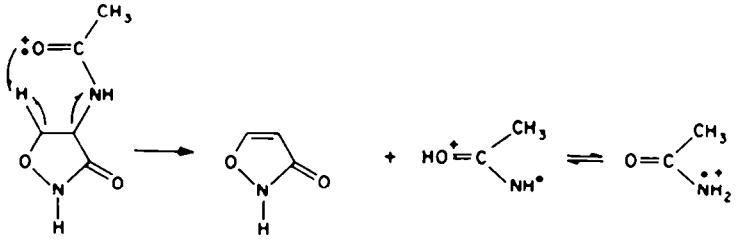
The fragmentation pattern of N-acetyl-d_3-cycloserine (XI, R = H) shows a very abundant ion at m/e 46 corresponding to CD_3CO^+ and a rather less abundant one at m/e 44, presumably corresponding to CD_2CO^+.



XI

The molecular ion from III loses ketene very rapidly to give an ion at m/e 144 and the corresponding metastable ion[33,34] at m/e 111·5 (calc., m/e 111·3). That it is the ring acetyl group that is so expelled, presumably in a four-center process,[33] giving the ion at m/e 144—which is actually the molecular ion from II—is suggested by the similarity below m/e 144 of the spectra of II and III and confirmed by the fragmentation of XI (R = $COCH_3$) which proceeds via the loss of ketene, with the expected metastable ion m/e 114·4, rather than the loss of ketene-d_2.

Loss of the side chain acetyl group of II as neutral ketene gives a fragment of m/e 102 which would be expected to carry a substantial proportion of the charge. As has been noted, however, the ion at m/e 102 is of low abundance and this is evidently because it is unstable and collapses rapidly to an ion of m/e 59, to judge by a prominent metastable ion at m/e 34·3 (calc. for m/e 102 → m/e 59; m/e 34·2). The accurate mass to charge ratio of this ion is 59·0349 which serves to identify it as $C_2H_5NO^+$ (m/e 59·0371). It seems therefore that this ion could be $CH_3CONH_2^+$, formed by elimination of the entire side-chain together with one hydrogen atom probably from position 5 according to the mechanism below; but this possibility



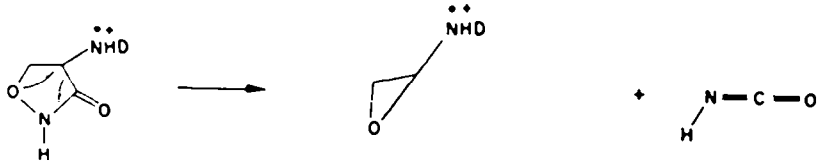
m/e 59

has to be discounted in view of the observation that replacement of this acetyl group by acetyl-d_3 causes a shift of the ion not to m/e 62, but to m/e 60. Clearly then, two deuterium atoms fail to survive this fragmentation, the first step of which must therefore be loss of ketene-d_2. This leads to the ion at m/e 103 which is of very low

[33] J. H. Beynon, *Mass Spectrometry and its Applications to Organic Chemistry* pp. 251–262. Elsevier, New York, N.Y. (1960).

[34] K. Biemann, *Mass Spectrometry* p. 154. McGraw-Hill, New York, N.Y. (1962).

abundance because it collapses rapidly as shown below to give the ion at m/e 60 together with a metastable ion at the calculated position of m/e 35·0.

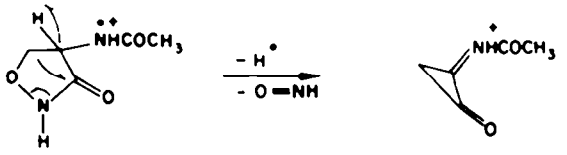


m/e 103 m/e 60 (43)

This "cross-ring" cleavage of five-membered heterocyclic rings has been observed elsewhere[35,36] and may well be of general value in the mass spectrometry of compounds containing such systems.

The remaining abundant ion which appears in the spectra of II and III is at m/e 112. This ion is presumably formed by collapse of the ion at m/e 144 since a metastable ion is observable in both spectra at the calculated position of m/e 87·1. Replacement of the side-chain acetyl group in II or III by acetyl-d_3 results in a shift of the ion at m/e 112 to m/e 115 and of the metastable ion to m/e 90·0 and permits the conclusion that the intact acetyl side-chain is present in the daughter ion. The accurate mass to charge ratio of the daughter ion in the spectrum of II is 112·040 which allows the assignment of the formula $C_5H_6NO_2^+$ (m/e 112·0398) to this ion.

In the light of such evidence, the following fragmentation may be postulated as a route whereby $C_5H_6NO_2^+$ may be formed from the molecular ion of II and, if correct, constitutes a further example of the aforementioned "cross-ring" cleavage.[35,36]



Further support for this general scheme of fragmentation may be derived from the mass spectrum of N-acetyl-2-methylcycloserine (VII), shown in Fig. 4. The

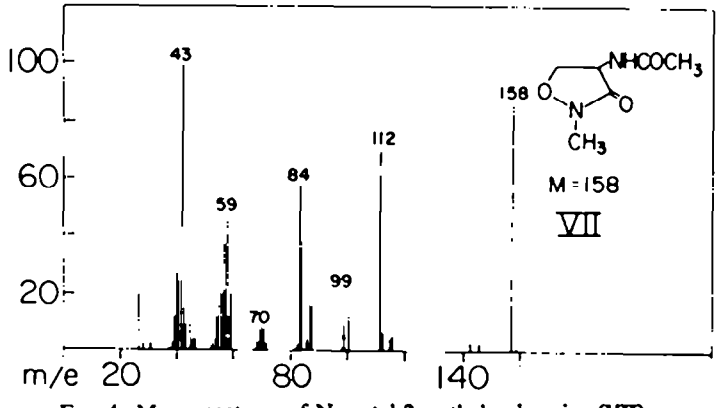


FIG. 4. Mass spectrum of N-acetyl-2-methylcycloserine (VII).

[35] A. Senning, *Chem. Comm.* **1**, 561 (1965).
[36] D. L. Klayman and G. W. A. Milne, *J. Org. Chem.* **31**, 2349 (1966).

molecular ion at m/e 158 loses CH_2CO or CH_3CO^+ giving small peaks at m/e 116 and m/e 115 and the base peak of the spectrum at m/e 43. The ion at m/e 116 presumably collapses readily to give that at m/e 59—a metastable ion being observed at the calculated position of m/e 30·0.

The ion at m/e 112 is observed in this spectrum and this fact tends to support the hypothesis previously advanced with respect to its structure and formation. The metastable ion accompanying its formation is found to be exactly as calculated, at m/e 79·4.

EXPERIMENTAL[87]

N-Acetylcycloserine (II) was prepared according to the method of Kuehl[10] and obtained as white needles, m.p. 179–180° (lit.[10] 175–177°).

N-Acetyl-2-methylcycloserine and N-acetyl-O-methylcycloserine were prepared by the action of diazomethane on N-acetylcycloserine as described by Hidy *et al.*[8] and separated by chromatography on Florisil with benzene as eluant. N-Acetyl-2-methylcycloserine was obtained in 41% yield as plates, m.p. 105–106° (lit.[8] 111–113°). N-Acetyl-O-methylcycloserine (VIII) was obtained in 37% yield as needles, m.p. 137–138° (lit.[8] 140–142°).

N,2-Diacetylcycloserine (III). Acetic anhydride (1·10 ml) was added to a solution of N-acetylcycloserine (1·44 g) in pyridine (30 ml, dried over CaH_2). The solution was allowed to stand at room temp for 45 min and then evaporated to dryness *in vacuo* to give a gum which was induced to crystallize upon trituration with ether. Three recrystallizations from acetone afforded chunky needles of III (1·35 g, 73%) m.p. 121–122° (lit.[9,11] 120–121°).

N-Acetyl-O-p-toluenesulfonylcycloserine (IX). N-acetylcycloserine (2·14 g) was dissolved in 5% $NaHCO_3$ aq (90 ml) and stirred at room temp during the rapid addition of a solution of *p*-toluenesulfonyl chloride (3·87 g) in acetone (45 ml). The mixture was stirred for 2 hr at 25° and then the acetone was removed under red. press. and the residual solution diluted with water. This solution was extracted with ethyl acetate (3 × 200 ml) and ether (200 ml). The organic extracts were combined, washed with water and dried ($MgSO_4$). Removal of the solvents *in vacuo* left a white solid which was recrystallized four times from ethyl acetate–benzene to give *N-acetyl-O-p-toluenesulfonylcycloserine* as soft needles (1·83 g, 42%), m.p. 149–150° dec. (Found: C, 48·11; H, 4·95; N, 9·29; S, 10·31. $C_{13}H_{14}N_2O_6S$ requires: C, 48·32; H, 4·73; N, 9·39; S, 10·75%.)

Hydrolysis of IX. N-Acetyl-O-*p*-toluenesulfonylcycloserine (IX, 29·8 mg) was dissolved in MeOH (15 ml) and to this solution was added 5·0 ml of 0·2M KOH aq (10·0 equiv.). After the solution had been stored for 2 hr at room temp, the pH was adjusted to 6·8 with 50% AcOH and an aliquot was subjected to high voltage electrophoresis (1500 V, pH 6·5) together with authentic *p*-toluenesulfonic acid. The hydrolysate was found to contain *p*-toluenesulfonic acid as its only mobile component detectable with the fluorescein spray that was used.

N-Acetyl-d_3-cycloserine (XI, R = H) was prepared according to the method of Kuehl[10] using acetic anhydride-d_6 in place of acetic anhydride and was obtained as white needles, m.p. 177–179°, undepressed by admixture with II.

N-Acetyl-d_3-2-acetylcycloserine (XI, R = $—COCH_3$) was prepared from XI (R = H) by the method used for the preparation of III, with acetic anhydride-d_6 in place of acetic anhydride, and was obtained as white needles, m.p. 120–122°, undepressed by admixture with III.

[87] All m.ps. were taken on a Kofler block and are uncorrected. Microanalyses were performed by Dr. W. C. Alford and his associates of this Institute.