Hydrolysis of Diacetylcycloserine. *In Vitro* and *in Vivo* Studies

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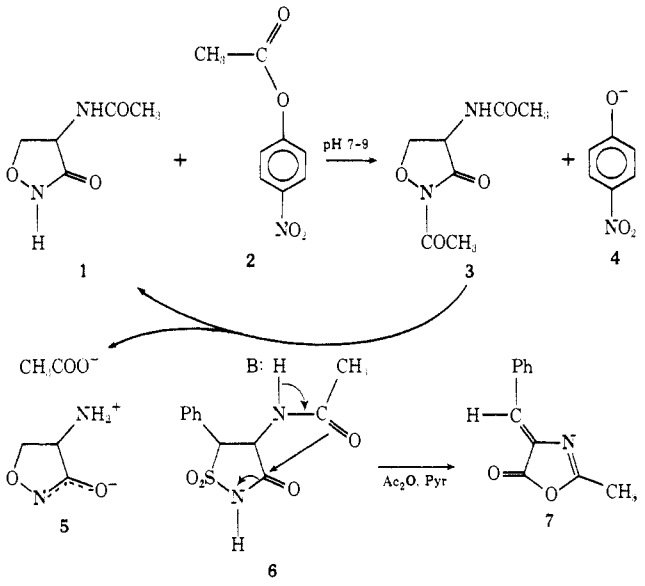
It is demonstrated that diacetylcycloserine (D-2-acetyl-4-acetamido-3-isoxazolidone, **3**) is hydrolyzed to α-*N*-acetyl-β-acetylaminooxy-D-alanine (**9**) under basic conditions. This indicates that the α-*N*-acetylcycloserine (**1**) catalyzed hydrolysis of *p*-nitrophenyl acetate (**2**) probably proceeds *via* general base catalysis rather than by way of **3** as had been previously postulated. However, when **3** was injected into rats it was not converted to **9** but was excreted as **1**. The reason for this difference between the *in vitro* and *in vivo* behavior of **3** is not known. The reaction of **3** with methanol and *n*-butylamine gave the α-*N*-acetyl-β-acetylaminooxy-D-alanine methyl ester (**11**) and *n*-butyl amide (**8**), respectively.

Since its isolation and characterization in 1955,[1,2] the broad spectrum antibiotic cycloserine (D-4-amino-3-isoxazolidone, **5**) has been the subject of continued interest directed toward an understanding of its biological activity and its relationship to the chemistry of the isoxazolidone ring system.[3,4] An interesting result of one chemical study was the finding that **5** and α-*N*-acetylcycloserine (**1**) catalyzed the hydrolysis of *p*-nitrophenyl acetate (**2**).[5,6] On the basis of kinetic experiments it was proposed that in the case of **1** this catalysis was the result of the transfer of the acetyl group of **2** to **1** yielding *p*-nitrophenol (**4**) and diacetylcycloserine (D-2-acetyl-4-acetamido-3-isoxazolidone, **3**) which then rapidly hydrolyzed to yield acetate and **1** (Scheme I).

Scheme I



It was also suggested that **1** might assist in other acyl transfer reactions in a manner similar to imidazole.[7]

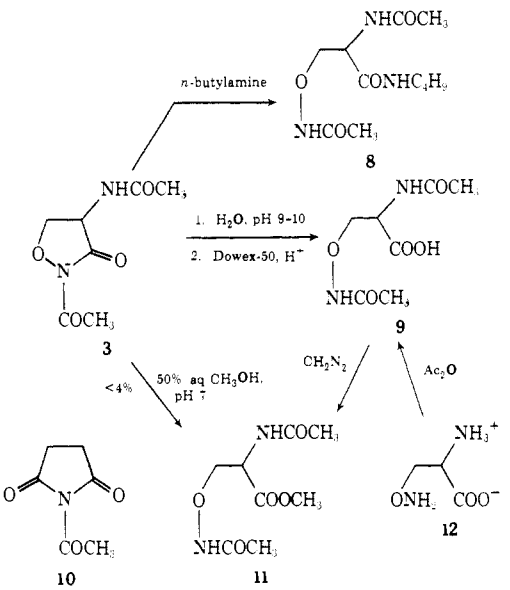
We became interested in these compounds indirectly. During an investigation of the chemistry of 4-acetamido-5-phenyl-3-isothiazolidinone 1,1-dioxide (**6**) we attempted acetylation using pyridine and acetic anhydride at room temperature, conditions used by other workers to synthesize **3** from **1**.[7] To our surprise, the product was 4-benzylidine-2-methyl-2-oxazolin-5-one (**7**) probably resulting from acetylation followed by base-catalyzed attack on the ring carbonyl by the exocyclic amide function (Scheme I) and subsequent elimination of acetamidosulfurous acid.[8]

These results prompted us to look more critically at the data used to support the suggested mechanism for the α-*N*-acetylcycloserine (**1**) catalyzed hydrolysis of **2**.[6] We found that there was no real evidence that diacetylcycloserine (**3**) was formed at all; the rate of the α-*N*-acetylcycloserine catalyzed hydrolysis was assumed to be the rate of synthesis of **3**, but it was not identified as the product. The hydrolysis of **3** was studied separately using material prepared by the cited method, but **1** was not identified as a product, and its rate of formation was merely assumed to be the same as the rate of hydrolysis of **3**.

It thus appeared that a reinvestigation of these reactions might be worthwhile chemically, and, since acetylation is a common detoxication mechanism, such a study might provide some useful information concerning cycloserine metabolism.

Initially we attempted to isolate **3** from hydrolysis reaction mixtures of **1** and **2**, prepared as described by Whish and Viswanatha.[6] When these attempts proved unsuccessful we shifted our emphasis to the hydrolysis of **3**. Paper chromatographic analysis of hydrolysis mixtures at pH 7–9 showed no evidence of **1**. The first clue as to the nature of the reaction came from studies carried out in 50% aqueous methanol at pH 7. A crystalline solid was obtained which infrared spectra and elemental analysis suggested was α-*N*-acetyl-β-acetylaminooxy-D-alanine methyl ester (**9**) (Scheme II). An unequivocal synthesis from β-aminooxy-D-alanine (**12**) *via* acetic anhydride and diazomethane confirmed the structure. The hydrolysis was then carried out in water and 50% aqueous acetone with the pH maintained at 9–10 by addition of sodium hydroxide. The reaction consumed approximately 1 equiv of base. The fact that only 1 equiv of acid was formed indicated that **3** was not hydrolyzing to **1** (p*K* 5.80)[1] and acetic acid (p*K* 4.76). This was confirmed by the isolation in high yield of the

Scheme II



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extremely hygroscopic product which was shown to be α-*N*-acetyl-β-acetylaminooxy-D-alanine (**9**) by elemental analysis, titration data, and unequivocal synthesis from β-aminooxy-D-alanine (**12**, Scheme II).

These results demonstrate that at pH 9–10 the hydrolysis of **3** proceeds mainly (and perhaps entirely) by ring cleavage rather than deacetylation. Since **1** is not regenerated by the hydrolysis of **3**, the mechanism for the hydrolysis of **2** represented in Scheme I cannot be operating to a significant extent at this pH. General base catalysis by the ionized form of **1** appears to be a more reasonable interpretation of its rate-accelerating effect; this is supported by the pH–rate profile which is consistent with the participation of the conjugate base of an acid possessing a p*K* similar to 1.[6]

We have not determined whether the hydrolysis of **3** proceeds through an oxazolidone intermediate similar to **7** or results from direct attack by hydroxide ion on the cyclic carbonyl.

The fact that **3** is hydrolyzed to **9** prompted us to look for it in the urine of rats which had been administered either **1**, **3**, or **5**. Our rationale for this was based on the following considerations. (a) The fate of about 30% of ingested cycloserine is unknown.[9] (b) Although **1** is not excreted after cycloserine ingestion, it could be that it was formed but was rapidly acetylated to **3** which in turn was hydrolyzed and excreted as **9**. (c) Since **9** is a new compound, it could have been overlooked in previous analyses of urine from patients or animals taking cycloserine.

Accordingly, **1**, **3**, and **5** were administered to rats and their urine was collected and analyzed by paper and gas–liquid chromatography. It was found that 80–100% of the administered **1** and **5** was excreted in the urine within 24 hr. Surprisingly, 80–100% of the administered **3** was excreted as **1** within the same period.

These results confirm that acetylation plays no important role in the cycloserine metabolism of the rat, a conclusion in agreement with previous studies. However, the finding that **3** is converted to **1** *in vivo*, while of no clinical significance, is interesting from a chemical viewpoint. Since **9** is the proven hydrolytic product at pH 9, and no evidence of **1** was found when **3** was hydrolyzed at pH 7, **3** must have undergone a qualitatively different reaction *in vivo*. Boyd and coworkers[10] have recently reported that *N*-acetylsuccinimide (**10**) acetylates amino groups much faster than it undergoes base-catalyzed hydrolytic ring cleavage. The structural similarity between **3** and **10** is obvious, and it seemed possible that **3** could also rapidly acetylate amino groups. We found, however, that *n*-butylamine reacted with **3** in water, alcohol, and ether to yield the ring-cleaved derivative, α-*N*-acetyl-β-acetylaminooxy-D-alanine *n*-butyl amide (**8**). Thus, the reason for this interesting difference in the *in vitro* and *in vivo* behavior of **3** remains unexplained.

Experimental Section

Melting points were observed in a calibrated Mel-Temp apparatus. Infrared spectra were recorded as films or mineral oil mulls with a Perkin-Elmer 137-B spectrophotometer. Glc data were obtained with a F & M 810 chromatograph using a 72 × 0.25 in. column and a 60–80 mesh acid-washed Chromosorb W support coated with 6% diethylene glycol succinate. Specific rotations were measured with a Rudolph Model 80 polarimeter. Elemental analyses were performed by Galbraith Laboratories, Knoxville Tenn., and agreed with the theoretical values to within ±0.4%. Cycloserine was generously given to us by the Commercial Solvents Co., Terre Haute, Ind.; α-*N*-acetylcycloserine was prepared by the method of Kuehl.[11]

Diacetylcycloserine (3). To a solution of 10.2 g (0.10 mol) of cycloserine in 500 ml of methanol was added 100 ml of acetic anhydride. The mixture was stirred at 25° for 3 hr and then evaporated *in vacuo* at 40° to a viscous oil which solidified when stirred with ether. The solid was collected, washed with ether, and dried in a desiccator over NaOH. The yield was 17.0 g (91%), mp 122–123°; recrystallization from 75 ml of ethanol raised the melting point to 123–124° (lit.[7] mp 121–122°), $[\alpha]^{25}D$ +64° (*c* 5, CH_3OH). The infrared spectrum and mixture melting point were identical with **3** prepared by the method of Milne and Cohen.[7]

α-*N*-Acetyl-β-acetylaminooxy-D-alanine (9, from 3). To a solution containing 0.93 g (5.0 mequiv) of **3** in 50 ml of 50% aqueous acetone (v/v) was added 1.0 *N* NaOH at a rate which kept the pH at 9.0–9.7 (combined glass-calomel electrode). After 1 hr 4.7 ml of base had been added and the reaction appeared complete. The sodium ions were removed with Dowex-50 and the solution was evaporated *in vacuo*. The residue (1.03 g, 100%) was triturated with ether and decanted, and the solid was dried *in vacuo*: mp 55–60° (noncrystalline); neut equiv, 214 (calcd 204); $[\alpha]^{25}D$ +9.2° (*c* 10, H_2O). *Anal.* ($C_7H_{12}N_2O_5$) C, H, N. The infrared spectrum was identical with **9** prepared from acetic anhydride and β-aminooxy-D-alanine and with the product of the hydrolysis carried out at the same pH in water alone.

Isolation of Diacetyl-β-aminooxy-D-alanine Methyl Ester (11) from the Hydrolysis of Diacetylcycloserine (3) in 50% Aqueous Methanol. (Although impractical as a preparative method this experiment is summarized here because it resulted in the first indication that ring cleavage was taking place.) A solution of 1.86 g (0.010 mol) of **3** in 100 ml of 50% aqueous methanol was kept at pH 7 for 10 min and then evaporated to dryness *in vacuo*, the residue was extracted, and the extracts were reevaporated using first acetone, then ethyl acetate. Extraction of the second residue with anhydrous ethyl ether and storage overnight at 4° afforded colorless crystals, mp 99–100°. Recrystallization from ethyl ether raised the melting point to 101–102°. The yield was less than 100 mg (4%). *Anal.* ($C_8H_{14}N_2O_5$) C, H, N. The melting point, mixture melting point, and infrared spectrum were identical with **11** prepared from authentic β-aminooxy-D-alanine by the acetic anhydride–diazomethane route.

Synthesis of α-*N*-Acetyl-β-acetylaminooxy-D-alanine (9) and α-*N*-Acetyl-β-acetylaminooxy-D-alanine Methyl Ester (11) from β-Aminooxy-D-alanine. To a solution of 0.24 g (2.0 mmol) of β-aminooxy-D-alanine (**12**)[12] in 40 ml of methanol was added 2.0 ml (20 mmol) of acetic anhydride. The mixture was stirred overnight at 25° and evaporated *in vacuo* to a gum which gave an infrared spectrum (film) insignificantly different from the product of hydrolysis of **3** in 50% aqueous acetone. The gum was dissolved in 4 ml of methanol and 1.7 mequiv of ethereal diazomethane[13] was added. The mixture was swirled in a hood for 15 min and then evaporated to dryness. The residue was dissolved in 8 ml of ethanol and filtered. About 150 ml of anhydrous ether was added and the solution was stored overnight at 4°. The colorless crystals which separated weighed 0.161 g (58%), mp 100–101°, $[\alpha]^{25}D$ +32° (*c* 3, CH_3OH).

α-*N*-Acetyl-β-acetylaminooxy-D-alanine *n*-Butyl Amide (8). To a chilled solution of 5.0 ml (0.050 mol) of *n*-butylamine and 10 ml of H_2O, 1.86 g (0.010 mol) of **3** was added gradually over a 2-min period. The solution was stored at 25° for 30 min and then evaporated under reduced pressure at 40–50°. A damp solid was formed which smelled strongly of butylamine. After storage overnight in a hood a friable solid remained: 2.45 g; mp 130–135°; preliminary mp 60–65°. Recrystallization from 10 ml of ethyl acetate gave 2.14 g (72%), mp 140–145°, preliminary mp 60–65°. Further recrystallization raised the final melting point to 165–166° but did not alter the infrared spectrum. The product was the dihydrate, and the preliminary melting point at 60–65° corresponded to the loss of $2H_2O$: $[\alpha]^{25}D$ +32° (*c* 6, CH_3OH). *Anal.* ($C_{11}H_{21}N_3O_4 \cdot 2H_2O$) C, H, N.

Animal Experiments. For each experiment three male albino rats weighing 400–500 g were used; each rat was kept in a separate metabolic cage. Water and food were supplied *ad libitum*. Before the administration of the drugs, urine was collected for 24 hr as the control sample. After drug administration rat urine was collected for three consecutive 24-hr periods. All samples were collected in an ice bath and then stored at −20° for chromatographic analysis. Each group of three rats was subjected to only one drug. The administration of **3** and **5** was carried out in three separate experiments; for **1**, two separate experiments were run. Cycloserine (**5**) was administered orally to the rats by stomach tube in dosages for 30, 120, and 180 mg per kg of body weight per day. Fresh solutions were prepared daily and administered at 10 a.m., 4 p.m., and 10 p.m. The concentration of the solutions was adjusted so that the volume of each dose was less than 1.1 ml.

For the administration of **1**, a 100 mg/ml aqueous solution was freshly prepared and injected into the rat tail vein in two incre-

Table I. Chromatographic Properties of Cycloserine and Several Derivatives

Compd	R_f Solvent A	R_f Solvent B	Color formed 0.4% $FeCl_3$	Color formed 4% Nitroprusside
1	0.7	0.7	Brown	Blue
3	0.8	0.8		
5	0.3	0.15	Brown	Blue
9	0.7	0.7	Pink	Blue

ments (at 10 a.m. and 4 p.m.) of 120 mg/kg (total dosage 240 mg/kg/day).

For the injection of 3, a fresh solution containing 180 mg/ml in 7% ethanol was prepared and a total dosage of 600 mg/kg/day was injected through the tail vein in increments of 300 mg/kg twice per day, at 10 a.m. and 4 p.m.

Chromatographic Analysis of Rat Urine Samples. 1. Paper Chromatography. Samples were spotted 2.5 cm away from each other and 3 cm from the bottom of a 28.5 × 42.0 cm sheet of Whatman No. 1 filter paper which was developed ascendingly with either 1-propanol-water (5:1, v/v, solvent A) or 1-butanol-acetic acid-water (4:1:1, v/v, solvent B). The two most useful detection reagents were 4% aqueous sodium nitroprusside[14] and 0.4% aqueous ferric chloride. Iodine vapor and uv light were used to detect 3. The R_f values of 1 and 9 were so similar that their relative migratory rates often reversed from experiment to experiment. This did not present a major problem because standards were always used and 1 and 9 gave different colored spots with $FeCl_3$. The chromatographic properties of 1, 3, 5, and 9 are presented in Table I.

After it had been determined by the use of $FeCl_3$ that 1 was being excreted in the urine of rats which had been administered 3, nitroprusside was used to obtain a semiquantitative value.

Semiquantitative Estimation. The percentages of administered compounds which were excreted as 1 or 5 could be estimated by comparison of the size and intensity of spots resulting from the chromatography of known amounts of 1 and 5 with spots derived from urine samples. The total amounts of 1 and 5 excreted in the 24-hr urine sample were then calculated from the total volume of urine excreted in this time. Since it was found that 5 μg of 5 and 10 μg of 1 could be detected by sodium nitroprusside, it can be calculated that less than 12% of the administered 5 was excreted as 1, and less than 10% of 1 was excreted as 5. There is no evidence that any interconversion of this type occurred; these calculations merely show that a small amount of 5 → 1 and 1 → 5 could occur without detection.

2. Gas-Liquid Chromatography. Although the results of paper chromatography indicated that the rat transformed 3 to 1 rather than to 9, more evidence that 9 was not being formed was desirable. This was obtained by treatment of rat urine samples (after preliminary column chromatography using Amberlite GC-400 resin to remove interfering substances) with diazomethane to convert any 9 which was present to the methyl ester 11. This was then subjected to glc. Control experiments in which 9 was carried through the same procedure indicated that 1 mg could easily be detected. When 50-ml urine samples from rats treated with 1, 3, or 5 were analyzed by this technique, no evidence of 11 was found.

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Angiotensin-Like and Antagonistic Activities of N-Terminal Modified [8-Leucine]angiotensin II Peptides†

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Two series of peptides related to [Ile^5]angiotensin II (AII) were synthesized by the solid-phase method: a Phe^8 series, composed of AII, [Suc^1]AII, des-Asp^1-AII, and des-Asp^1,Arg^2-AII; and a Leu^8 series, composed of [Leu^8]AII, [Suc^1,Leu^8]AII, des-Asp^1-[Leu^8]AII, and des-Asp^1,Arg^2-[Leu^8]AII. The AII-like and AII-antagonistic activities of these peptides were studied on the isolated guinea-pig ileum, rat uterus, and rabbit aorta and on the rat blood pressure. Comparison of pA_2, pD_2, and intrinsic activities of the two series of peptides led to the conclusion that [Leu^8]AII, [Suc^1,Leu^8]AII, and des-Asp^1-[Leu^8]AII antagonized AII by binding on the same receptor site, but it is suggested that a different site is involved in the agonistic activities of these peptides. Conclusions were also drawn on the importance of the N-terminal end for binding and for the intrinsic activity at the two postulated receptor sites.

Several angiotensin II analogs with aliphatic amino acids in position 8 of the peptide chain have been synthesized and shown to be specific competitive angiotensin antagonists.[2] Among the most potent of these inhibitors is [Leu^8]angiotensin II,[2b,d] which, however, possesses small angiotensin-like agonistic activities. It was thought of interest to study structure-activity relationships of 8-leucine angiotensin peptides, both as agonists and antagonists, and to compare these relationships with those observed

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