AZIRINOMYCIN. II
ISOLATION AND CHEMICAL CHARACTERIZATION AS 3-METHYL-2(2H) AZIRINECARBOXYLIC ACID

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Azirinomycin is a new antibiotic and is the first example of a natural product containing an azirine ring. It was isolated by ion-exchange and solvent extraction and is unstable, especially in concentrated form. It was identified as 3-methyl-2(2H) azirinecarboxylic acid by spectral measurements of its methyl ester and by identification of L-α-aminobutyric acid as a hydrogenation product.

Culture filtrates[4] containing azirinomycin were studied. Although the antibiotic is unstable in aqueous or solvent solutions, concentrates of sufficient purity for structure studies were obtained. These studies and comparison of spectral properties of the methyl ester with reported azirines[2,3] indicate that azirinomycin is 3-methyl-2(2H) azirinecarboxylic acid.

This represents the first reported example of the occurrence of an azirine ring in a natural product. This compound is produced in relatively high concentration (280 μg/ml) and hence must be synthesized at a rapid rate by the producing culture.

Materials and Methods

Assays

Samples were assayed by the disc-plate agar diffusion method using *Proteus vulgaris* MB-838 as the test organism. Pure methyl ester gave a 25 mm zone with 1/2″ discs at about 1 mg/ml. Since the esterification reduced the activity by half, it was assumed that pure azirinomycin is twice as active as its ester. Assays were then recalculated based on these figures.

Stability

The bioactivity in filtered broth was shown to be very unstable under alkaline conditions and somewhat unstable at pH 3 or pH 7, however, activity was retained at pH 5 for at least 2 days at room temperature. Concentrates prepared by the isolation methods described below proved to be very unstable. If the oily residue from concentration of ethyl acetate extracts was allowed to warm to room temperature at the end of the operation, darkening and loss of bioactivity occurred. If the residue was dissolved in water, adjusted to pH 5 and stored overnight, refrigerated or frozen, the solution became alkaline, darkened and lost bioactivity. Samples of extract were preserved for a few days by dissolving the residue in methylene chloride and storing the solution at dry-ice temperature.

Isolation

Fermentation broth from shake flasks was filtered through a pad of filter-aid and 3.4 liters of filtrate was adsorbed on 200 ml of Dowex 1×2 chloride cycle resin at 20 ml/min. The adsorbate was washed with water and eluted with 3 % aqueous NaCl. The first 100 ml of eluate was discarded and the next 200 ml was collected. Assays indicated a yield of 90 %.

The fraction from above was adjusted to pH 3.0 with HCl and passed over 50 ml of Dowex 50×2 sodium cycle resin to remove some impurities. The first 50 ml of effluent was discarded and the next 250 ml was collected. Bioassays indicated a yield of 65 %. This fraction was adjusted to pH 3 and extracted three times with equal volumes of ethyl acetate. The extracts were dried with Na_2SO_4, combined and concentrated under reduced pressure to an oil. Bioassays indicated a yield of 50 % over extraction and concentration. The product was soluble in methylene chloride, alcohols, water and ethyl acetate but insoluble in carbon tetrachloride or hexane.

The average yield of oil for several batches was 165 mg/liter of filtered broth and later analyses indicate this product was about 50 % pure. Since the yield by bioassay was 30 %, the broth contains about 280 mg/liter of antibiotic. Further purification was unsuccessful because of instability.

The sodium salt was prepared by diluting the product with water and titrating the solution with dilute NaOH. One of the most potent samples exhibited a pH 1/2 of 3.6 and an equivalent weight of 100. This sample was about 50 % pure and was tested in mice[4].

Methyl Ester

The product from ethyl acetate extraction was treated with portions of diazomethane in ether until the reaction mixture was neutral. The amount of diazomethane required indicated an equivalent weight of 140 for the mixture. The solution was then concentrated to an oil. Two combined batches (6.5 liters of filtered broth) yielded 1.4 ml of oil.

Gas-liquid Chromatography

The crude ester was purified by GLC in a Varian Aerograph 200 equipped with a 6′ ×1/4″ column of Gas-Chrom P loaded with 4 % ethylene glycol succinate polyester. The column was operated at 140°C with an inlet temperature of 160°C and the retention time of the major component was 7 minutes. The product was collected by inserting a small glass tube into the exit as a condenser. Ten 100 μl portions were chromatographed and yielded 371 mg of pale yellow liquid.

Hydrogenation

A 150 mg sample of oil from the extraction of the antibiotic was dissolved in methylene chloride and added to a suspension of 0.1 g of pre-reduced PtO_2 in the same solvent. The mixture was shaken under 1 atmosphere of H_2 pressure and 19 ml of hydrogen was absorbed in 15 minutes. After 30 minutes the catalyst was filtered off and washed with methylene chloride. The filtrate and washings were concentrated to dryness and yielded 35 mg. The cake was washed with methanol and yielded 51 mg of partly crystalline material. This residue was recrystallized from methanol and yielded 5 mg of product which was identified as α-aminobutyric acid by microanalysis, IR and NMR spectra. The methanol mother liquors were concentrated to dryness, dissolved in 0.2 ml of water and crystallized by adding 4 ml of ethanol. An additional 5.3 mg of amino acid was obtained and was shown to be the L-isomer by ORD. The value observed was $[\phi]=+2{,}150°$ at 225 nm in 0.5 M HCl while the literature value is $[\phi]=+2{,}460°$ at 225 nm in 0.1 M HCl[1].

A sample of 2 mg of the pure methyl ester was hydrogenated in ethanol with PtO_2 catalyst. The product was examined by TLC and gave a positive ninhydrin spot whose mobility was greater than that of α-aminobutyric acid, however, after saponification with NaOH, the sample exhibited one ninhydrin-positive spot with the same mobility as an adjacent sample of α-aminobutyric acid.

Thin-Layer Chromatography

For the analysis of the antibiotic or its hydrogenation product, plates coated with Avicel microcrystalline cellulose (Brinkman Instruments, Inc., Westbury, N.Y.) were used and *n*-butyl alcohol - acetic acid - water (3 : 1 : 1) was used as developer. Spots were revealed with ninhydrin, brom-thymol blue, iodine vapor or bioautograpy. The eluate from Dowex 1×2 showed minor ninhydrin spots at Rf values of 0.26 and 0.39 and major com-

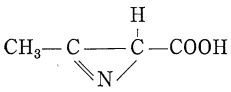
ponents at 0.47 and 0.82. Bioautographs showed that the fastest spot is the antibiotic. In the Dowex 50 adsorption step all of the Rf 0.47 material is removed along with some of the two minor components. Hydrogenation of the Dowex 50 effluent, containing the antibiotic, eliminated the 0.82 spot and produced a spot with an Rf value of 0.31, identical to the mobility of α-aminobutyric acid. Elution of the Dowex 50 with 0.2 M NH_3 yielded a sample of the 0.47 material which when chromatographed after hydrogenation gave no ninhydrin reaction at all. Thus the α-aminobutyric acid must be produced from the antibiotic.

The spot at Rf value 0.47 was also found in purified samples which had partially decomposed, thus, this compound is probably a decomposition product of the antibiotic.

Results and Discussion

The structure of azirinomycin was deduced from the structures of its hydrogenation product and methyl ester. Catalytic hydrogenation of crude isolates destroyed the bioactivity and yielded L-α-aminobutyric acid. Examination of the crude by TLC indicated that this compound was produced from the antibiotic rather than from an impurity.

Fig. 1. Structure of azirinomycin



The IR spectrum of crude antibiotic in chloroform solution showed typical COOH bands at 2400~3500 cm^{-1} and an absorption band at 1800 cm^{-1}, suggesting a strained ring carbonyl. The spectrum of the pure ester showed a band at 1725 cm^{-1} and no active hydrogen absorpion, consistent with a methyl ester. Weaker bands were found at 1790, 1665 and 1565 cm^{-1}. The band at 1790 is typical of azirines[3].

The NMR spectrum of pure methyl ester in $CDCl_3$ showed: a three-proton singlet at 6.27 τ; a three-proton singlet at 7.42 τ; and a one-proton singlet at 7.56 τ. These resonances are in agreement with those reported for 3-methyl-2-carbethoxy-2H-azirine[2].

These spectral meaurements suggest that the antibiotic is a methylazirinecarboxylic acid. Since the methyl signals observed in the NMR are not split and the amino acid isolated from the hydrogenation is optically active, the antibiotic is 3-methyl-2(2H)-azirinecarboxylic acid.

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