[CONTRIBUTION FROM JOHN L. SMITH MEMORIAL FOR CANCER RESEARCH]

E-73: An Antitumor Substance. Part II. Structure[1]

BY KOPPAKA V. RAO

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E-73 is an anti-tumor substance produced by the organism *Streptomyces albulus*. It is accompanied by the known antifungal substance cycloheximide. From a study of the acid and alkaline degradation products, E-73 is shown to be 4-acetoxycycloheximide.

E-73 (I) is a colorless crystalline solid with anti-tumor activity isolated from the culture filtrate of *Streptomyces albulus*.[2] It is accompanied in the broth by two diastereoisomeric forms of cycloheximide (II). In some of its physical properties as shown in Figs. 1 and 2, E-73 resembles cycloheximide closely. This similarity suggested that E-73 may be structurally related to cycloheximide.

The structure of cycloheximide was elucidated by the elegant work of Kornfeld, Parke and Jones.[3] One of the key reactions in this work was alkaline hydrolysis whereby were obtained the volatile 2,4-dimethylcyclohexanone, ammonia and an aldehydic acid which could be oxidized to methanetriacetic acid.

E-73 has a molecular formula $C_{17}H_{25}O_6N$. It forms a monoacetate and a mono-*p*-nitrobenzoate thus showing the presence of a hydroxyl group. Formation of a monosemicarbazone and quantitative oximation demonstrate the presence of a single carbonyl group. Preliminary alkaline hydrolysis gives rise to one mole of ammonia. However, in sharp contrast with cycloheximide, no volatile ketone is obtained. Also acidification and distillation of the hydrolysis mixture gives rise to acetic acid.

These products do not give much insight into the structure, and acid hydrolysis is studied next. Graded acid hydrolysis gives three crystalline products representing three stages of the reaction. At room temperature, with 6 *N* acid, are obtained acetic acid and a product $C_{15}H_{23}O_5N$ (III). This latter compound is very similar to E-73 in its physical properties and could be reacetylated with some difficulty to give acetyl E-73. This is, therefore, designated as desacetyl E-73. Either E-73 (I) or desacetyl E-73 (III) on brief treatment with hot acid gives rise to a second product $C_{15}H_{19}O_3N$ (IV) which is called desacetyl dehydro E-73. All three of these on prolonged acid hydrolysis give the final product, $C_{15}H_{20}O_5$ (V), called desacetyl dehydro E-73 acid. Both desacetyl dehydro E-73 (IV) and the final product V resemble each other but differ sharply from E-73 (I) and desacetyl E-73 (III). They (IV and V) both exhibit a characteristic ultraviolet absorption spectrum with a maximum at 280 mμ (ϵ 2000) in contrast to the other two which give a value of 20. They also contain a hydroxyl group but no carbonyl function. Desacetyl dehydro E-73 (IV) can be shown to be an imide and the final product V the corresponding acid.

No information is available regarding the action of acid on cycloheximide (II) under these conditions. This reaction is, therefore, studied for comparison. Under mild conditions a crystalline product is obtained whose properties showed that it is the already known anhydro cycloheximide (VI). This was previously made by Kornfeld, Parke and Jones[3] by the action of phosphorus pentoxide on cycloheximide. Further acid hydrolysis yields the corresponding dicarboxylic acid VII (ϵ_{max} 9000 at 241 mμ). Both these compounds VI and VII show characteristic properties of α,β-unsaturated ketones in contrast to desacetyl dehydro E-73 (IV) and the corresponding acid V which show properties characteristic of phenolic compounds. Thus, cycloheximide loses one mole of water to yield α,β-unsaturated carbonyl compounds while E-73 loses two moles of water to yield phenolic products. Hence, if desacetyl E-73 has the same carbon skeleton as cycloheximide, it must have at least one of its hydroxyls located in the cyclohexanone part of the molecule. Strong support for this assumption is obtained from the conversion of cycloheximide itself into desacetyl dehydro E-73 (IV) by the action of N-bromosuccinimide. Bromination is followed by rapid dehydrobromination and under the acid conditions dehydration also takes place. The unstable intermediate thus formed rearranges to give desacetyl dehydro E-73 (IV). This reaction definitely establishes that both E-73 and cycloheximide have the same carbon skeleton. It is also clear that E-73 is acetoxycycloheximide.

In order to define the exact location of the acetoxy group in E-73, the alkaline hydrolysis is studied further. After hydrolysis with alkali and continuous extraction with ether, a relatively non-volatile and water-miscible ketone (VIII) is obtained. This has a molecular formula of $C_8H_{14}O_2$ and its ultraviolet and infrared spectra are compatible with those of a hydroxy-2,4-dimethylcyclohexanone. This ketone is resistant to periodate oxidation thus showing that the hydroxyl does not occupy positions 2 or 6. The ketone can be readily dehydrated in presence of acid to yield an α,β-unsaturated ketone. This same compound can also be obtained from 2,4-dimethylcyclohexanone by bromination and dehydrobromination. Although these reactions add strong support for the ketone to be a hydroxydimethylcyclohexanone, they cannot establish definitely the position of the hydroxyl group in the ketone because of the possibility of double bond migrations during the dehydration step.

(1) Presented at the 134th Meeting of the American Chemical Society, Chicago, Ill., September, 1958.

(2) Koppaka V. Rao and Walter P. Cullen, THIS JOURNAL, **82**, 1127 (1960).

(3) E. C. Kornfeld, R. G. Jones and T. V. Parke, *ibid.*, **71**, 150 (1949).

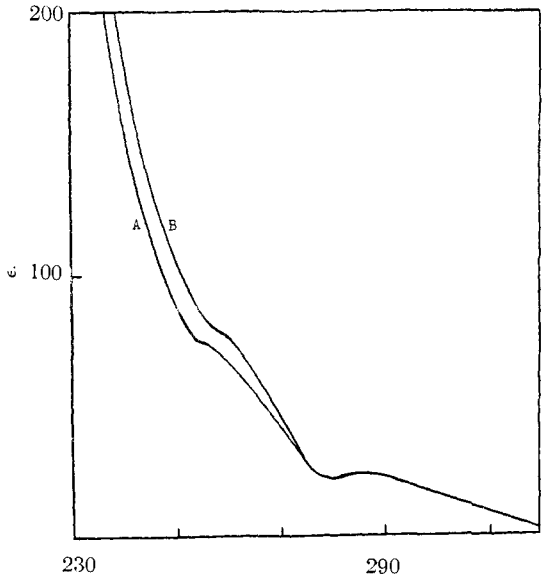


Fig. 1.—Ultraviolet absorption spectra of E-73 (A) and cycloheximide (B).

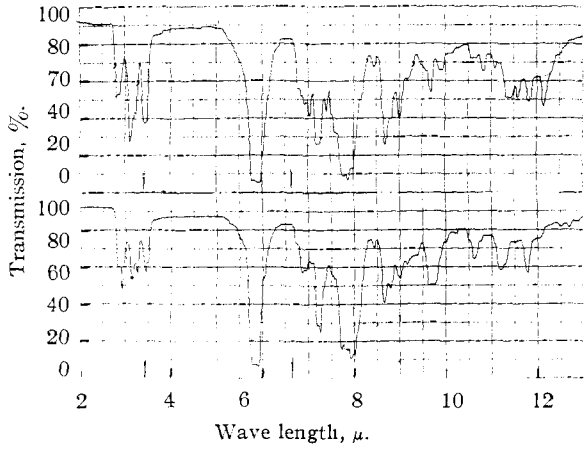


Fig. 2.—Infrared spectra of E-73 (upper curve) and cycloheximide (lower curve).

That the hydroxyl group does, indeed, occupy the 4-position can be shown by quantitative oxidation studies with chromic acid. Under the conditions of the reaction, secondary cyclohexanols consume one mole of oxygen almost instantaneously and further oxidation is rather slow and gradual (Fig. 3). Cyclohexanones undergo non-specific oxidation at a slow rate, one mole of oxygen being consumed in 1–2 hours. On the other hand, cyclohexanones with a β-hydroxyl group (such as cycloheximide) react vigorously and a rapid degradation follows. Against this background of the three possible types, the oxidation of the ketone from E-73 is studied. It parallels the reaction with 2,4-dimethylcyclohexanone with only a gradual consumption of oxygen. This provides substantial proof that the hydroxyl is tertiary. Additional evidence was also supplied by nuclear magnetic resonance spectrum kindly supplied by Professor E. J. Corey of the University of Illinois.

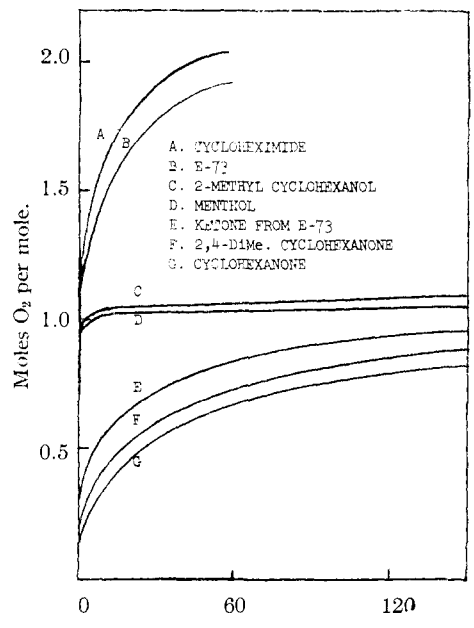


Fig. 3.—Chromic acid oxidations.

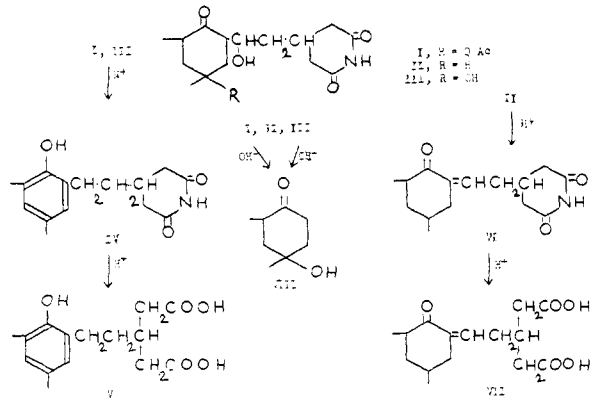


Fig. 4.—Degradation of E-73.

E-73 thus is assigned the structure I and the reactions are shown in Fig. 4[4a,b]

Experimental

E-73.—For purification, E-73 is crystallized two or three times from a mixture of ethanol and ether (1:4). When crystallized from ethanol alone, E-73 separates out as colorless transparent needles which melt at 65–67°. This form is stable for several months if left at about 5°. However, if left at room temperature for a day or two it changes to colorless opaque crystals which melt at 141–142°. The higher melting form is obtained directly when E-73 is crystallized from solutions which contain diethyl ether. The product is sparingly soluble in ether and benzene, moderately in water and lower alcohols and readily in acetone, chloroform and methylene chloride; $[\alpha]_D$ −8.8° (*c* 1% in methanol). *Anal.* Calcd. for $C_{17}H_{25}O_6N$: C, 60.18; H, 7.37; N, 4.13; acetyl, 12.68; mol. wt., 339. Found: C, 60.48; H, 7.64; N, 4.44; acetyl, 8.6; mol. wt. (acetone), 312, 349.

E-73 Acetate.—E-73 (0.3 g.) was dissolved in a mixture of acetic anhydride (2.0 ml.) and dry pyridine (0.5 ml.).

(4) (a) In passing it might be noted that inactone which is a dehydro cycloheximide isolated by R. Paul and S. Tchelitcheff, *Bull. soc. chim. France*, 1316 (1955), is a close relative of cycloheximide and desacetyl E-73. (b) A recent report by R. R. Herr, THIS JOURNAL, **81**, 2595 (1959), concerning the structure of the streptovitacins has shown that the previously reported desacetyl E-73 (III) and streptovitacin A are the same.

After the solution was left overnight, the reagents were removed in a current of air and residue crystallized from a mixture of methylene chloride and ether. The acetyl derivative separates out as colorless shining needles which melt at 177–178°. *Anal.* Calcd. for $C_{19}H_{27}O_7N$: C, 59.83; H, 7.14; N, 3.67. Found: C, 59.89; H, 7.17; N, 3.76.

E-73 *p*-Nitrobenzoate.—A solution E-73 (0.35 g.) in dry pyridine (2 ml.) was treated with *p*-nitrobenzoyl chloride (0.11 g.). The mixture was heated on a steam-bath for 10 minutes and treated with dilute hydrochloric acid. The solid which separated out was filtered, washed with water and crystallized from a mixture of methylene chloride and ether. The product separates out as colorless thin rectangular plates which melt at 167–168°. *Anal.* Calcd. for $C_{24}H_{28}O_9N_2$: C, 59.01; H, 5.78; N, 5.74. Found: C, 58.57; H, 5.78; N, 5.86.

E-73 Semicarbazone.—To a mixture of semicarbazide hydrochloride (1 g.) and sodium acetate (2 g.) in water (5 ml.) was added a solution of E-73 (0.5 g.) in ethanol (2 ml.). When the clear solution was left for several hours at room temperature the semicarbazone separated out as a colorless crystalline solid. It was filtered, washed and recrystallized from methanol whereby it separated out as colorless rectangular prisms, m.p. 214–215°. *Anal.* Calcd. for $C_{18}H_{26}O_6N_4$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.20; H, 7.37; N, 14.14.

For quantitative oximation, a weighed amount of E-73 was refluxed with a solution of hydroxylamine hydrochloride and pyridine and the acid liberated was titrated; calcd. CO, 8.23; found CO, 7.88.

Desacetyl E-73 (III).—E-73 (0.5 g.) was dissolved in 6 *N* hydrochloric acid (10 ml.). The solution was left at room temperature for 30 minutes and then diluted with water (30 ml.). Any precipitate that separated out was filtered off and the filtrate extracted with three portions of methylene chloride. Concentration of the combined solvent extracts gave a colorless crystalline solid. When recrystallized from methylene chloride it separated out as colorless needles, m.p. 164–165°, yield 0.15 g. *Anal.* Calcd. for $C_{15}H_{23}O_5N$: C, 60.59; H, 7.80; N, 4.71. Found: C, 59.99; H, 7.48; N, 4.19.

A portion of desacetyl E-73 (0.1 g.) was acetylated by heating with acetic anhydride (2 ml.) and pyridine (0.5 ml.) at 70° for 2 hours and leaving at room temperature for 24 hours. Repeated crystallization of the product gave colorless needles which melted at 178–180° alone or in admixture with acetyl E-73.

Acid Hydrolysis of E-73 to Compound IV.—E-73 (1 g.) was dissolved in 6 *N* hydrochloric acid (10 ml.) and the clear solution heated at 80–90° for 3–5 minutes. A colorless crystalline solid separated out which was filtered, washed and recrystallized from aqueous methanol; yield 0.5 g., m.p. 147–148°. *Anal.* Calcd. for $C_{15}H_{19}O_3N$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.43; H, 7.52; N, 5.67. The same substance can also be obtained by the following procedures:

(a) A solution of E-73 (0.34 g.) in isopropyl alcohol (3 ml.) was treated with 48% hydrobromic acid (0.2 ml.). The solution was warmed gently and let stand for 3 hours. It was diluted with water and extracted with methylene chloride twice. Evaporation of the solvent extract gave a colorless crystalline solid which melted at 147–148°. Its mixed melting point with the HCl-hydrolyzed product was not depressed and the infrared spectrum was identical with that of the latter.

(b) Cycloheximide (II) (1.4 g.) was dissolved in methylene chloride (25 ml.) and the solution refluxed with N-bromosuccinimide (1 g.) for 20–30 minutes in presence of a 200 watt GE sun lamp. A deep yellow color developed soon and became discharged in a few minutes. At the end of 20–30 minutes there was a negative test for N-bromosuccinimide (starch–iodide paper). The reaction mixture was shaken with water and the solvent extract concentrated to dryness. A colorless glassy residue was obtained which slowly evolved hydrogen bromide fumes. It was dissolved in pyridine (15 ml.) and the solution refluxed for 2 hours. It was cooled, poured over crushed ice and treated with sulfuric acid until slightly acidic. It was then extracted three times with methylene chloride and the combined solvent extracts concentrated to dryness. A light gray glassy solid was obtained which was purified by passing an ether solution of it through a column of acid-washed alumina. Evaporation of the effluent gave a colorless crystalline solid which melted at 147–148°. Its mixed melting point with HCl-hydrolyzed E-73 was not depressed; yield 0.6 g.

The substance prepared by the above procedures is sparingly soluble in water but readily so in common organic solvents. Acetylation with acetic anhydride and pyridine gave a colorless crystalline acetyl derivative which was recrystallized from ether; m.p. 130–132°. *Anal.* Calcd. for $C_{17}H_{21}O_4N$: C, 67.31; H, 6.98; N, 4.62. Found: C, 66.74; H, 6.99; N, 4.66.

Acid Hydrolysis of E-73 to Compound (V).—E-73 (4 g.) was refluxed with 6 *N* hydrochloric acid (100 ml.) for 4 hours. The colorless crystalline solid which separated out in a few minutes dissolved slowly and a colorless oil separated out. The mixture was cooled after 4 hours and the crystalline solid filtered and washed; yield 2.6 g. It was recrystallized from aqueous methanol whereby it separated out as colorless glistening rectangular plates, m.p. 140–141°. *Anal.* Calcd. for $C_{15}H_{20}O_5$: C, 64.28; H, 7.14. Found: C, 64.25; H, 7.43.

Acetylation of the above substance with acetic anhydride and pyridine at room temperature yielded a colorless crystalline derivative, m.p. 124–126°. *Anal.* Calcd. for $C_{17}H_{22}O_6$: C, 63.34; H, 6.88. Found: C, 63.04; H, 6.92.

For methylation, the above sample (0.5 g.) in acetone (50 ml.) was refluxed with dimethyl sulfate (1.5 ml.) and anhydrous potassium carbonate (5 g.) for 12 hours. The mixture was filtered, the cake was washed with acetone and the filtrate concentrated to remove most of the solvent. The oily residue was refluxed with 5% aqueous sodium hydroxide for one hour and acidified. The precipitate was recovered and crystallized from aqueous methanol, m.p. 116–117°. *Anal.* Calcd. for $C_{16}H_{22}O_5$: C, 65.29; H, 7.53. Found: C, 65.21; H, 7.58.

The filtrate from the acid hydrolysis was extracted continuously with ether for 24 hours. The extract was concentrated in presence of water and the aqueous concentrate neutralized and refluxed with a slight excess of *p*-bromophenacyl bromide. The ester was crystallized from aqueous methanol. It separated out colorless plates melting at 85–86°. Mixed melting point with *p*-bromophenacyl acetate was undepressed and the infrared spectra were identical.

The hydrolysis mixture from E-73 was distilled with excess of aqueous sodium hydroxide and the distillate collected in 1 *N* hydrochloric acid. The distillate was concentrated to dryness and the product crystallized from methanol acetone. *Anal.* Calcd. for NH_4Cl: N, 26.17; Cl, 66.28. Found: N, 26.95; Cl, 66.10.

Alkaline Hydrolysis of E-73 to 2,4-Dimethyl-4-hydroxycyclohexanone (VIII).—E-73 (1.0 g.) was let stand overnight in 10% aqueous sodium hydroxide (50 ml.). It was then extracted repeatedly with equal volumes of ether. Concentration of the combined extract gave a colorless oil (0.2 g.). It distilled at 130–135° (bath temperature) at 0.3 mm. The ketone was obtained as a colorless hygroscopic oil with a faint odor. *Anal.* Calcd. for $C_8H_{14}O_2$: C, 67.57; H, 9.93. Found: C, 66.71; H, 10.06; $[\alpha]_D$ +7.1° (*c* 1% in methanol), $n^{25}D$ 1.4656. The ketone is readily soluble in water. A portion of the ketone was converted into the 2,4-dinitrophenylhydrazone which was crystallized from a mixture of ether and isopropyl ether. The product separated as lemon-yellow rectangular plates, m.p. 130–132°. *Anal.* Calcd. for $C_{14}H_{18}O_5N_4$: C, 52.17; H, 5.63; N, 17.38. Found: C, 51.91; H, 5.85; N, 17.27.

A portion of the above ketone (0.05 g.) was distilled with 6 *N* hydrochloric acid. The distillate was treated with 2,4-dinitrophenylhydrazine. The product when recrystallized from a mixture of methylene chloride and ether separated out as maroon-red needles, m.p. 178–180°. *Anal.* Calcd. for $C_{14}H_{16}O_4N_4$: C, 55.28; H, 5.29; N, 18.39. Found: C, 54.84; H, 5.52; N, 18.39.

2,4-Dimethylcyclohexanone (0.1 g.) obtained from cycloheximide according to the procedure of Kornfeld, Parke and Jones[2] was refluxed with a slight molar excess of N-bromosuccinimide in methylene chloride (15 ml.) in the presence of a 200 watt GE sunlamp. At the end of the reaction the mixture was washed with water, dried and refluxed with pyridine (5 ml.) for 2 hours. The solution was diluted with water, acidified to *p*H 2.0 and extracted with ether. The solvent extract was treated with 2,4-dinitrophenylhydrazine. The product crystallized from methylene chloride–ether as maroon-red needles, m.p. 178–180°. Mixed melting with the corresponding sample obtained from 2,4-dimethyl-4-hydroxycyclohexanone was not depressed and

their infrared spectra were identical. *Anal.* Calcd. for $C_{14}H_{16}O_4N_4$: C, 55.28; H, 5.29; N, 18.39. Found: C, 55.05; H, 5.17; N, 18.36.

Anhydrocycloheximide (VI).—Cycloheximide (1 g.) was dissolved in 6 *N* hydrochloric acid (15 ml.) and the solution maintained at 80–90° for about 5 minutes. The crystalline solid which separated out on cooling was filtered and recrystallized from aqueous methanol. The product separated out as glistening colorless rectangular plates, m.p. 133–135° (reported[2] m.p. for anhydrocycloheximide 134–135°). *Anal.* Calcd. for $C_{15}H_{21}O_3N$: C, 68.41; H, 8.04; N, 5.32. Found: C, 68.92; H, 8.17; N, 5.48.

Anhydrocycloheximide Acid (VII).—Anhydrocycloheximide (0.5 g.) was refluxed with 6 *N* hydrochloric acid for 1 hour. The mixture was cooled and extracted with ether. The acid VII was obtained as a colorless glassy solid and hence it was characterized as the following salts. A solution of the acid in ether was treated with benzylamine in ether until there was no more precipitate. The benzylamine salt was crystallized from acetone–ethyl acetate. It separated out as colorless rectangular prisms, m.p. 153–154°. *Anal.* Calcd. for $C_{29}H_{40}O_5N_2$: C, 70.13; H, 8.12; N, 5.64. Found: C, 70.57; H, 7.98; N, 5.66.

The above benzylamine salt was dissolved in water and passed through Amberlite IR-C50 which is converted into the barium form. The effluent and the wash were concentrated to dryness and the product crystallized from aqueous methanol. The barium salt separated out as colorless glistening plates which decomposed at 275–280° (ϵ 9000 at 241 mμ). *Anal.* Calcd. for $C_{15}H_{20}O_5Ba$: C, 43.15; H, 4.82; Ba, 32.88. Found: C, 43.58; H, 4.83; Ba, 32.79.

Acknowledgment.—The author is grateful to Dr. R. L. Wagner for analyses and to Dr. F. A. Hochstein for many helpful suggestions.

MAYWOOD, N. J.

[CONTRIBUTION FROM THE ORGANIC CHEMISTRY DEPARTMENT, RESEARCH DIVISION, ABBOTT LABORATORIES]

Synthesis of Potential Diuretic Agents. I. Derivatives of 7-Sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-Dioxide

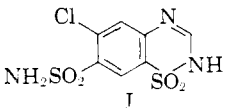
BY W. J. CLOSE, LEO R. SWETT, LEONARD E. BRADY, JAMES H. SHORT AND MAYNETTE VERNSTEN

RECEIVED JUNE 11, 1959

The synthesis of several new 7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxides from substituted 4-amino-1,3-benzenedisulfonamides is described. Ring closure was effected with formaldehyde as well as with higher aldehydes and halogenated aldehydes.

The need for better diuretic and hypotensive agents is attested by the extensive research effort which is being expended in these areas. Although a great variety of drugs is now available to the physician, most of these substances have severe limitations that impair their usefulness.

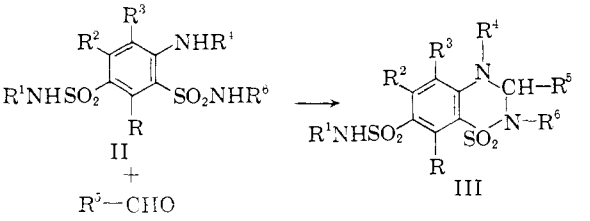
Recently, derivatives of 1,2,4-benzothiadiazine 1,1-dioxide have been shown in experimental animals to be carbonic anhydrase inhibitors, and to produce marked diuresis.[1-4] One member of this class (chlorothiazide, I) has achieved wide clinical acceptance as a diuretic and hypotensive agent.



I

In a systematic study of compounds related to chlorothiazide, we discovered that some dihydro compounds in the same series are considerably more effective in experimental animals than the aromatic substances.[5,6] This report describes the synthesis of compounds belonging to this class.

7-Sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxides (III) were prepared by condensation of substituted 4-amino-1,3-benzenedisulfonamides (II) with aldehydes, usually in aqueous solution.[7] The intermediate disulfonamides were generally prepared in the classical manner through chlorosulfonation followed by treatment with ammonia.



II + R^5—CHO → III

Most of the compounds prepared were derived from 4 - amino - 6 - chloro - 1,3 - benzenedisulfonamide, but compounds containing fluoro, bromo, methyl, trifluoromethyl, methoxy and nitro groups were also made. Simple aliphatic and halogenated aliphatic aldehydes were used in the cyclization. The products are high-melting solids which tend to decompose at elevated temperatures. They are insoluble in water and acid, but soluble in caustic solutions, from which they can be recovered unchanged by acidification. Boiling with alkali readily opens the thiadiazine ring with the formation of the original disulfonamide.

Special consideration was given to the preparation of N-alkylated products. Chlorosulfonation of methylaniline was used to prepare 4-methylamino-1,3-benzenedisulfonamide, from which a 4-methyl derivative (III, $R^4 = CH_3$) was obtained. A compound in which both sulfonamide nitrogens carry a methyl substituent (III, $R^1 = R^6 = CH_3$; $R^2 =$

(1) F. C. Novello and J. M. Sprague, THIS JOURNAL, **79**, 2028 (1957).

(2) R. F. Pitts, F. Krück, R. Lozano, D. W. Taylor, O. P. A. Heidenreich and R. H. Kessler, *J. Pharmacol. Exptl. Therap.*, **123**, 89 (1958).

(3) K. H. Beyer, *Ann. New York Acad. Sci.*, **71**, 363 (1958).

(4) J. M. Sprague, *ibid.*, **71**, 328 (1958).

(5) Since this work was begun, we have learned that other groups have independently carried out investigations on similar compound. Compare G. de Stevens, L. H. Werner, A. Halamandaris and S. Ricca, Jr., *Experientia*, **14**, 463 (1958); W. Kobinger and F. Lund, *Ugeskrift Laeger*, **120**, 1583 (1958); C. T. Holdredge, R. B. Babel and L. C. Cheney, Abst. 135th National Meeting Am. Chem. Soc., p. 19N (1959); and L. H. Werner, A. Halamandaris, S. Ricca, Jr., L. Dorfman, and G. de Stevens, *ibid.*, p. 27N (1959); J. E. Baer, H. F. Russo, and K. H. Beyer, *Proc. Soc. Exptl. Biol. Med.*, **100**, 442 (1959).

(6) Preliminary pharmacological data on the compounds described in this report were presented by M. E. Goldberg and K. Hwang, *Federation Proc.*, **18**, 396 (1959).

(7) J. H. Freeman and E. C. Wagner, *J. Org. Chem.*, **16**, 815 (1951).