[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

# Structural Studies on the Antibiotic Netropsin

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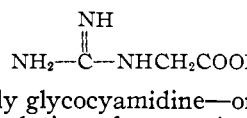
The antibiotic netropsin is a diacidic base possessing the molecular formula $C_{18}H_{26}N_{10}O_3$ and is convertible by hydrolysis to one mole each of ammonia, guanidinoacetic acid and the monobasic netropsinine ($C_{15}H_{20}N_6O_3$).

Several years ago Finlay, Hochstein, Sobin and Murphy[1] described the isolation and characterization of a new antibiotic, Netropsin,[2] obtained from the culture filtrates of the Actinomycete *Streptomyces netropsis*. Since the publication of the early work, we have had the opportunity to reinspect the chemical nature of the antibiotic; the present communication describes its further characterization as well as the results of certain preliminary degradation studies.

Mainly on the basis of the analysis of the sulfate, the approximate molecular formula $C_{32}H_{48}N_{18}O_4$ was assigned to netropsin by the earlier workers. While securing additional analytical data, we observed that the sulfate was hygroscopic and seemingly firmly solvated, and, depending on the extent of drying, the nitrogen analyses, for example, varied between 24 and 26.5%. We found, however, that the limiting values obtained on exhaustively dried portions of the sulfate were fairly constant and indicated the formula $C_{18}H_{26}N_{10}O_3 \cdot H_2SO_4$ (or somewhat better, $C_{18}H_{26}N_{10}O_3 \cdot {}^1/_2H_2O \cdot H_2SO_4$). This assignment was confirmed by analyses on the dipicrate and the dihelianthate of netropsin as well as by the additivity of the molecular formulas of the degradation products obtained on mild basic hydrolysis (*vide infra*). Netropsin sulfate gives a negligible quantity of acetic acid in the Kuhn–Roth determination for C-methyl groups.

Finlay, *et al.*, described the degradation of netropsin sulfate brought about by the neutralization with an equivalent amount of alkali, resulting in the formation of two *bases:* one, a water-soluble compound possessing the empirical formula $C_3H_5N_3O$; the second, a water-insoluble substance, the analysis of which indicated the composition $C_{15}H_{20}N_6O_3$. Repetition of the basic decomposition invariably gave, in our hands, a product $C_3H_7N_3O_2$, along with the $C_{15}$-base, the analysis of which we were able to confirm. The smaller fragment, obtained either by direct crystallization or through an ion-exchange procedure, has now been identified as *guanidinoacetic acid*. We can account for the isolation by the earlier workers of the product $C_3H_5N_3O$—probably glycocyamidine—only by assuming a milder degradation of netropsin sulfate than we normally effected, or, less likely, by cyclization of the guanidinoacetic acid during the isolation or purification process.



The $C_{15}$-base referred to above was characterized in this Laboratory as the monopicrate, m.p. 216°, and the monohydrochloride, m.p. 200° dec., as well as by a monobenzoyl derivative, m.p. 271°, and a diacetyl derivative, m.p. 157–158°. Electrometric titration of the free base ($pH_b$ 6.8) evidenced only one basic center, thereby making the equivalence of the molecular and empirical formulas for netropsinine virtually a certainty.

Since the decomposition affording guanidinoacetic acid and the $C_{15}$-base would in all probability constitute the starting point for subsequent structural studies, the reaction was studied in some detail. It was determined that neutralization of netropsin sulfate at room temperature liberated one mole of ammonia (observed value 0.96 mole) and that hydrazine, hydroxylamine and nitrogen were not formed in this operation. We were not successful in isolating any degradation products other than those indicated, all of which were formed in good yield.[1]

Drastic basic hydrolysis of netropsin sulfate, carried out by refluxing in excess aqueous sodium hydroxide for eighty hours, yielded 4.03 moles of ammonia, on the basis of the $C_{18}H_{26}N_{10}O_3$ formula. The assumption that guanidinoacetic acid should yield two moles of ammonia under similar hydrolysis conditions was confirmed by the observed value of 2.03 moles, and it was further determined that the $C_{15}$-base affords exactly one mole under such circumstances. Thus the number of moles of ammonia surrendered by the degradation products, taken together with the single mole liberated during the mild, basic fragmentation of netropsin sulfate, corresponds well with the total value liberated by netropsin sulfate itself.

During the course of this investigation, several observations of incidental interest were made. The addition of an equivalent amount of sodium methoxide in methanol to netropsin sulfate followed by brief heating served only to convert the antibiotic salt to the $C_{15}$-base; furthermore, the latter was stable to the same reagent on refluxing in methanol for short periods of time. In attempting to substantiate the report that netropsin sulfate gives a positive Ehrlich test, we noted that no color developed at room temperature, although warming resulted in a blue-violet, rather than the usual pink, coloration. Since netropsin is so readily cleaved by base, the positive Sakaguchi test afforded by the antibiotic[1] may well be due to guanidinoacetic acid which is liberated in the medium used for the test, *viz.*, excess aqueous alkali. Because of these complications, the deductions which can be made on

(1) A. C. Finlay, F. A. Hochstein, B. A. Sobin and F. X. Murphy, THIS JOURNAL, **73**, 341 (1951).

(2) Netropsin is the trademark of Chas. Pfizer and Co., Inc., for the antibiotic produced by *Streptomyces netropsis*.

the basis of these color tests are limited. Finally, the high degree of similarity between the ultraviolet spectra[1] of netropsin sulfate and the $C_{15}$-base signifies that the chromophoric system present in the former has been largely preserved during the basic degradation to the latter and that the change involved is therefore probably not a deep-seated one.

It is clear from the foregoing that netropsin is composed only of the guanidinoacetic acid and the $C_{15}H_{20}N_6O_3$ moieties, linked together with the incorporation of a molecule of ammonia, and that the structural problem involves in essence the nature of the $C_{15}$-base and its attachment to the other two components.

TABLE I

| Compound | Solvent | $\lambda_{max}$ mμ | log ε |
|---|---|---|---|
| Netropsin sulfate | 0.1 *N* HCl | 236, 297 | 4.30, 4.32 |
| $C_{15}$-base | 0.1 *N* HCl | 283 | 4.33 |
| | Ethanol | 243, 304 | 4.14, 4.33 |
| Monobenzamide of $C_{15}$-base | Ethanol | 234, 303 | 4.37, 4.48 |
| Diacetate of $C_{15}$-base | Ethanol | 240, 301 | 4.14, 4.23 |

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## Experimental

**Salts of Netropsin.**—For the analytical determinations, netropsin sulfate was repeatedly recrystallized from hot water, from which solvent, on cooling, it was deposited as well-formed, colorless needles. The individual samples were dried *in vacuo* at 80–125° to constant weight. A large number of analyses were obtained, and the values on samples dried at higher temperatures (100° and up) fell between narrow limits. The results cited below are the averages of the more satisfactory runs.

*Anal.* Calcd. for $C_{18}H_{28}N_{10}O_3 \cdot H_2SO_4$: C, 40.90; H, 5.30; N, 26.51; S, 6.06. Calcd. for $C_{18}H_{26}N_{10}O_3 \cdot {}^1/_2H_2O \cdot H_2SO_4$: C, 40.22; H, 5.44; N, 26.07; S, 5.96. Found: C, 40.70; H, 5.44; N, 25.87; S, 6.20.

Netropsin sulfate gave, in the Kuhn–Roth determination, 0.1% C-methyl.

**Netropsin picrate** was prepared by adding an excess of aqueous picric acid to a hot solution of purified netropsin sulfate in water. Since the picrate decomposed during attempted recrystallizations, it was merely dried to constant weight (56° *in vacuo*) and analyzed. The salt decomposes at 232°, after browning and sintering at about 225°.

*Anal.* Calcd. for $C_{18}H_{28}N_{10}O_3 \cdot 2C_6H_3N_3O_7$: C, 40.54; H, 3.60; N, 25.23. Found: C, 40.72; H, 3.64; N, 25.31.

**Netropsin helianthate**, obtained by the addition of a warm aqueous solution of methyl orange to a warm aqueous solution of netropsin sulfate, was purified by repeated crystallization from aqueous ethanol. The orange plates melted at 215° dec.

*Anal.* Calcd. for $C_{18}H_{26}N_{10}O_3 \cdot H_2O \cdot 2C_{14}H_{15}N_3O_3S$: C, 52.17; H, 5.52; N, 21.16. Found: C, 51.87; H, 5.47; N, 20.86.

**Isolation and Identification of Guanidinoacetic Acid.**—Netropsin sulfate (1.00 g.) was decomposed with an equivalent amount of alkali according to the previously published method.[1] The $C_{15}$-base, which precipitates out completely on standing overnight, was filtered off. The aqueous filtrate, which contained the guanidinoacetic acid, was applied to a column made up from Dowex-50 ion-exchange resin (acid form). On passing dilute hydrochloric acid through the column, guanidinoacetic acid was eluted as the hydrochloride; the qualitative detection was accomplished through tests using Sakaguchi reagent. The aqueous solution containing the hydrochloride was evaporated to a volume of several milliliters and applied to a water-washed column prepared from Dowex-1 in the basic phase. The free base could be eluted with water, and the solution thus obtained was evaporated to dryness at room temperature under reduced pressure, thereby yielding a colorless, crystalline product.

Guanidinoacetic acid also could be obtained by direct crystallization. The aqueous filtrate obtained, after removal of the $C_{15}$-base, by decomposition of 9.8 g. of netropsin sulfate, was reduced to a volume of 20 ml., filtered and allowed to stand in the refrigerator for 48 hours. The colorless, crystalline precipitate which had deposited was filtered off and washed successively with a small amount of water, ethanol and ether. After drying in a vacuum desiccator, the product weighed 1.08 g. (50% yield).

*Anal.* Calcd. for $C_3H_7N_3O_2$: C, 30.79; H, 6.03; N, 35.88. Found: C, 31.28; H, 6.06; N, 35.35.

The infrared spectra, measured in Nujol mull, of the above base and of authentic guanidinoacetic acid were indistinguishable.

The guanidinoacetic acid isolated as described above was converted to the picrate, m.p. 201° dec., and the hydrochloride, m.p. 189–190° dec. The melting points of the corresponding salts of authentic guanidinoacetic acid melt, respectively, at 201–202° dec. and 191° dec.[3] The appropriate mixed melting points showed no depression. To complete the identification, the guanidinoacetic acid obtained from netropsin was converted to hydantoinimine hydrochloride,[3] m.p. 209–210° dec. No depression in melting point was observed on admixture with authentic hydantoinimine hydrochloride, m.p. 211–212° dec. Again, the infrared spectra (mulls) of the two salts were identical.

**Determinations of Ammonia Released During Basic Hydrolyses. Neutralization of Netropsin Sulfate.**—One gram of *well-dried* netropsin sulfate was treated with 20 ml. of 0.103 *N* sodium hydroxide in a closed system. During a 24-hour period, a slow stream of nitrogen was passed through the reaction mixture, the ammonia entrained being trapped in 5% boric acid solution and titrated with standard hydrochloric acid (brom cresol green–methyl red indicator). A total of 0.96 mole of ammonia was detected.

Since no water-insoluble gas was liberated during the neutralization of netropsin sulfate, free nitrogen was not a degradation product. Furthermore, color tests with salicylaldehyde[4] demonstrated the absence of hydrazine or hydroxylamine among the hydrolysis products.

**Drastic Basic Hydrolysis of Netropsin.**—Using either excess aqueous 0.5 *N* barium hydroxide or sodium hydroxide, netropsin sulfate was hydrolyzed by refluxing until no more ammonia was released (60–80 hours of refluxing was required for completion). During the hydrolysis the ammonia was swept through with nitrogen and determined as described above. The highest value for moles of ammonia released was 4.03.

**Drastic Basic Hydrolysis of Guanidinoacetic Acid.**—Using the conditions described for netropsin, guanidinoacetic acid was hydrolyzed completely in 60–75 hours, affording 2.03 moles of ammonia.

**Drastic Basic Hydrolysis of the $C_{15}$-Base.**—Under similar circumstances, the $C_{15}$-base released exactly one mole of ammonia.

**Salts and Derivatives of the $C_{15}$-Base.**—As obtained from the aqueous basic decomposition of netropsin sulfate, the $C_{15}$-base is a pink microcrystalline solid, crystallizable from hot water, after which the melting point is 253–255° dec. The base also can be obtained by treatment of netropsin sulfate with an equivalent amount of sodium methoxide in methanol. After being heated for 5 minutes, ice was added. After reheating and subsequent cooling, the product precipitated as a pale pink powder. The analytical data on purified material substantiate the $C_{15}H_{20}N_6O_3$ formulation of Finlay, *et al.*[1]

**The picrate**, m.p. 216° dec., was secured through the use of aqueous picric acid.

*Anal.* Calcd. for $C_{15}H_{20}N_6O_3 \cdot C_6H_3N_3O_7$: C, 41.02; H, 3.32; N, 21.27. Found: C, 41.35; H, 3.97; N, 21.00.

(3) Korndorfer, *Arch. Pharm.*, **242**, 629 (1905); H. King, *J. Chem. Soc.*, 2375 (1930).

(4) F. Feigl, "Qualitative Analysis by Spot Tests," Nordemann Publishing Co., Inc., New York, N. Y., 1937, p. 149–152.

The hydrochloride, prepared by solution of the base in 0.1 *N* hydrochloric acid and subsequent evaporation in a desiccator, melted at 200° dec.

*Anal.* Calcd. for $C_{15}H_{20}N_6O_3 \cdot HCl$: C, 48.84; H, 5.74; N, 22.79; Cl, 9.61. Found: C, 48.81; H, 5.72; N, 22.71; Cl, 9.60.

**The monobenzamide** was prepared by adding 0.15 ml. of benzoyl chloride to 400 mg. of base suspended in pyridine (4.0 ml.). After standing for two hours, the reaction mixture was worked up by addition of 20 ml. of ether, which resulted in the deposition of a gummy solid. After being washed with ether and triturated with chloroform, the solid was crystallized from ethanol. The amide (230 mg.) was secured as colorless needles, m.p. 271°.

*Anal.* Calcd. for $C_{22}H_{24}N_6O_4$: C, 60.59; H, 5.53; N, 19.28. Found: C, 60.51; H, 5.50; N, 19.22.

**The diacetyl derivative** of the $C_{15}$-base was obtained by acetylation with isopropenylacetate in glacial acetic acid, the reaction mixture being refluxed for one hour. The product, isolated by ether precipitation, was recrystallized twice from ethanolic ether, after which it melted at 157–158°.

*Anal.* Calcd. for $C_{15}H_{18}N_6O_3(CH_3CO)_2$: C, 54.70; H, 5.80. Found: C, 54.78; H, 5.81.

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# Reactions of Ethylenimines. VIII. Dissociation Constants

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Ethylenimine, 2,2-dimethylethylenimine and 2-ethylethylenimine were found to be weak bases with $pK_B$ values of 5.99, 5.36 and 5.69, respectively.

In spite of the fact that ethylenimines(aziridines) have been known since 1888[1] no apparent attempt has been made to determine their basic strengths in aqueous solution. The problem was avoided in these laboratories for a long time because it appeared that hydrolysis would make such a measurement difficult by the usual techniques. But a hint was available in the literature[2] that the reaction with water was slow and our own recent kinetic work on the hydrolysis of ethylenimines[3] in acid made the measurement of base strength appear feasible.

The dissociation constants of ethylenimine, 2,2-dimethylethylenimine, 2-ethylethylenimine and three related amino alcohols have been determined by use of the glass electrode *p*H meter. While the dissociation constants measured thus and presented in Table I are not true thermodynamic values, the *relative* basicities may be determined quite precisely. In this actual case, the values of $K_B$ may be close to the thermodynamic values since the values of $K_a$ for the conjugate acids of the imines and amino alcohols were found to be independent of ionic strength in the range from 0.01 to 0.1. The constants obtained for the three amino alcohols compare favorably with the thermodynamic values given by Glasstone and Schram.[4]

TABLE I

DISSOCIATION CONSTANTS OF ETHYLENIMINES AND AMINOALCOHOLS AT 25°

| Compound | $pK_B$ (obsd.) | $K_B$ | $pK_B$ (lit.)[a] |
|---|---|---|---|
| Ethylenimine | 5.99 | $1.0 \times 10^{-6}$ | |
| 2-Ethylethylenimine | 5.69 | $2.0 \times 10^{-6}$ | |
| 2,2-Dimethylethylenimine | 5.36 | $4.3 \times 10^{-6}$ | |
| Ethanolamine | 4.56 | $2.8 \times 10^{-5}$ | 4.55 |
| 2-Amino-1-butanol | 4.45 | $3.3 \times 10^{-5}$ | 4.48 |
| 2-Methyl-2-amino-1-propanol | 4.29 | $5.3 \times 10^{-5}$ | 4.28 |

[a] Ref. 4.

The experimental value for the dissociation constant of ethylenimine is in accord with the prediction of Brown and Gerstein[5] that it would be weaker than dimethylamine ($K_B = 7.4 \times 10^{-4}$). The value for ethylenimine may also be considered to meet the qualification of "low basicity" mentioned by Searles, Tamres and Lippincott.[6] Furthermore, the value of $K_B$ for ethylenimine itself ($1.0 \times 10^{-6}$) is in agreement with the results of Barb[7] (7 to $9 \times 10^{-7}$), reported since this work was completed.

As may be seen in Table I, C-alkyl groups on ethylenimines have an effect of considerable size on the basicity of the ring nitrogen atom. The enhancement is an order of magnitude or so larger than would be expected for an inductive effect through a saturated carbon atom. It is of the same magnitude as the effect of 3- or 4-methyl groups in many aromatic systems, although it is less than that caused by a 4-methyl group in either pyridine or aniline.[8] It is well known that three-membered rings of the cyclopropane, ethylene oxide and ethylenimine types behave in a manner similar to an unsaturated system.[9] The large enhancement of nitrogen basicity by C-alkyl substitution which is described here seems to be an example of this "unsaturated character" of three-membered rings.

## Experimental

The ethylenimines were prepared by the method of Wenker[10] and distilled from sodium just prior to use. The index

(1) S. Gabriel, *Ber.*, **21**, 1049 (1888).

(2) H. Freundlich and W. Neumann, *Z. physik. Chem.*, **87A**, 69 (1914), and later papers of Freundlich.

(3) V. B. Schatz and L. B. Clapp, THIS JOURNAL, **77**, 5113 (1955).

(4) S. Glasstone and A. F. Schram, *ibid.*, **69**, 1213 (1947).

(5) H. C. Brown and M. Gerstein, *ibid.*, **72**, 2926 (1950).

(6) S. Searles, M. Tamres and E. R. Lippincott, *ibid.*, **75**, 2775 (1953).

(7) W. G. Barb, *J. Chem. Soc.*, 2564 (1955).

(8) A compilation of $pK_a$ values for aromatic systems may be found in D. H. McDaniel and H. C. Brown, THIS JOURNAL, **77**, 3756 (1955).

(9) Lists of pertinent references may be found in (a) N. H. Cromwell and M. A. Graff, *J. Org. Chem.*, **17**, 414 (1952); (b) N. H. Cromwell, N. G. Barker, R. A. Wankel, P. J. Vanderhorst, F. W. Olson and J. H. Anglin, THIS JOURNAL, **73**, 1044, 5929 (1951); (c) J. D. Roberts and V. C. Chambers, *ibid.*, **73**, 5030, 5034 (1951); (d) M. T. Rogers, *ibid.*, **69**, 2544 (1947).

(10) (a) H. Wenker, *ibid.*, **57**, 2328 (1935); (b) T. L. Cairns, *ibid.*, **63**, 871 (1941); (c) G. D. Jones, *J. Org. Chem.*, **9**, 484 (1944).