[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEARCH LABORATORIES, AMERICAN CYANAMID COMPANY]

Chemotherapeutic Dyes. V. Benzo[a]phenothiazines and Benzo[a]phenazines

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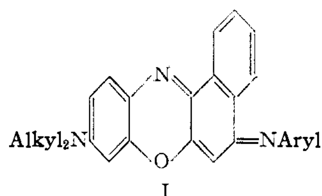
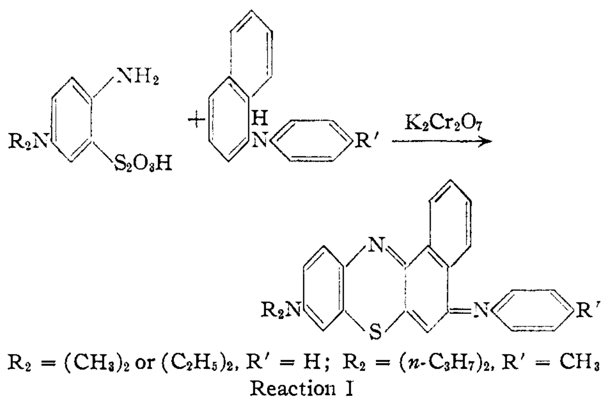
Three 9-dialkylamino-5-aryliminobenzo[a]phenothiazines and 9- and 10-diethylamino-5-phenyliminobenzo[a]phenazine were synthesized. The methods of preparation and the activity of these compounds in mouse tuberculosis in comparison with analogous benzo[a]phenoxazines were studied.

Benzo[a]phenoxazines of Formula I[1] have been shown to be highly effective in prolonging the survival time of mice infected with tuberculosis when administered orally.[2]



Along with this work a study of the structurally similar benzo[a]phenothiazines and phenazines was undertaken.

No benzo[a]phenothiazines substituted in the desired fashion have been reported. Methods[3] used for the preparation of 3,7-disubstituted phenothiazines of the methylene blue type were found to be suitable. The three benzo[a]phenothiazines were prepared by Reaction I.



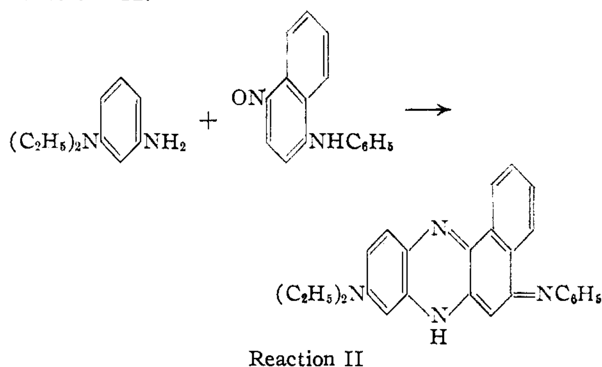
$R_2 = (CH_3)_2$ or $(C_2H_5)_2$, $R' = H$; $R_2 = (n\text{-}C_3H_7)_2$, $R' = CH_3$

Reaction I

The yield varied from 15 to 40% in this reaction. Purification of the compounds was effective only when they were handled as the free bases. The hydrochloride was useful in isolation but it could not be purified satisfactorily. The bases crystallized well from hydrocarbon solvents and after such treatment were chromatographically pure, as judged by examination on filter paper with hexane as a solvent. This technique proved quite useful in following the elimination of impurities.

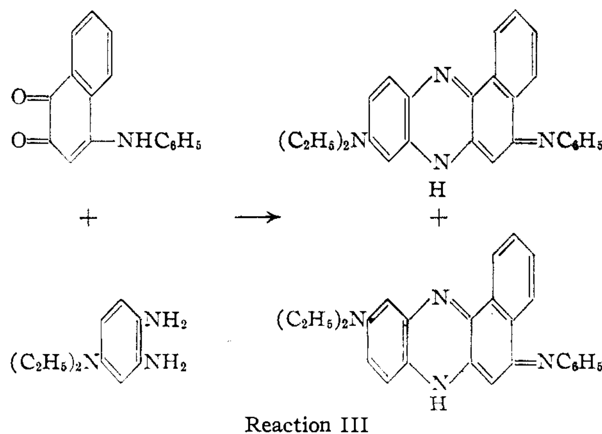
The dyes were quite similar to the corresponding benzo[a]phenoxazines, being blue in acid and red in basic solutions. The absorption maxima in acid of the benzo[a]phenothiazines are at 15–30 mμ longer wave lengths than the corresponding oxygen compounds.

The desired 5-phenylimino-9-diethylaminobenzo[a]phenazine was prepared in very small yield by Reaction II.



Reaction II

For preparative purposes it was necessary to proceed by a different reaction (Reaction III),[4] which gave rise to not only the desired 9-diethylamino compound but also its 10 isomer. The production of the same compound by Reactions II and III leaves no doubt as to the structure of the 9 isomer. The 9 and 10 isomers were isolated in yields of 17 and 45%, respectively, after purification.



Reaction III

The compounds were fairly easily separated due to the greater insolubility of the 10-isomer. The 9-isomer is the more strongly basic as might be expected, and is identical with the isolated product of Reaction II in all respects.

The compounds were tested for their effect on the survival time of tuberculous mice by administration in the diet in comparison with 9-diethylamino-5-phenyliminobenzo[a]phenoxazine[1,2,5] as a stand-

(1) M. L. Crossley, R. J. Turner, C. M. Hofmann, P. F. Dreisbach and R. P. Parker, THIS JOURNAL, **74**, 578 (1952).

(2) H. J. White, M. E. Schlosser and M. B. DiCenzo, paper presented to Society of American Bacteriologists, Chicago, Illinois, May, 1951.

(3) A. Bernthsen, *Ann.*, **251**, 91 (1889).

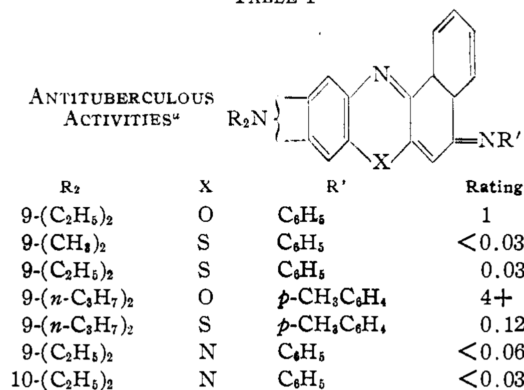
(4) F. Ullmann and J. Gnaedinger (*Ber.*, **45**, 3446 (1912)) report a similar reaction with *o*-phenylenediamine.

(5) We are indebted to Dr. H. J. White and his co-workers for this data.

ard. The results are shown in Table I. The substituents have the same relative effect in both the benzo[a]phenoxazine and benzo[a]phenothiazine series, but the most active compound in the latter is only one-sixtieth as active in this test as its oxygen analog. Whether this relative inactivity is due to lack of absorption of the compounds by the mice or is inherent is not known.

TABLE I

ANTITUBERCULOUS ACTIVITIES[a]



R_2	X	R'	Rating
9-$(C_2H_5)_2$	O	C_6H_5	1
9-$(CH_3)_2$	S	C_6H_5	<0.03
9-$(C_2H_5)_2$	S	C_6H_5	0.03
9-$(n\text{-}C_3H_7)_2$	O	$p\text{-}CH_3C_6H_4$	4+
9-$(n\text{-}C_3H_7)_2$	S	$p\text{-}CH_3C_6H_4$	0.12
9-$(C_2H_5)_2$	N	C_6H_5	<0.06
10-$(C_2H_5)_2$	N	C_6H_5	<0.03

[a] Determined by the methods used in M. J. Baker, M. E. Schlosser and H. J. White, *Ann. N. Y. Acad. Sci.*, **52**, 678 (1949). The rating is determined by the relative amount of drugs in the diet required to produce an equal effect on survival time. The ratings are reproducible within twofold limits. < means inactive as tested.

Experimental[6]

9-Diethylamino-5-phenyliminobenzo[a]phenazine.—A solution of 201 mg. (0.001 mole) of 3-amino-N,N-diethylaniline[7] and 372 mg. (0.0015 mole) of 4-nitroso-1-anilinonaphthalene[8] in 3 cc. of 90% acetic acid was allowed to stand at room temperature for 20 hours and then evaporated to dryness in a stream of air. The residue was dissolved in 7 cc. of alcohol, and 100 cc. of phosphate–citrate buffer at *p*H 3.5 was added. The solution was extracted with three 30-cc. portions of petroleum ether and the *p*H then raised to 7 by the addition of phosphate buffer. Five cc. of alcohol was added and the slurry was extracted once with petroleum ether.

The petroleum ether extracts were discarded.

The aqueous residue from the petroleum ether extract was extracted with 100 cc. of 1:1 ether–benzene. The extract was evaporated and the residue was dissolved in 40 cc. of 1:1 ether–petroleum ether and chromatographed on Filter Cel (25 × 180 mm.), developing with the same solvent until fluorescence appeared in the effluent. The solvent was changed to ether and development continued until a band was removed. The column was discarded. The residue from the evaporation of the ether effluent was dissolved in 30 cc. of 1:1 ether–petroleum ether and chromatographed on a 35 × 280 mm. alumina column, developing with ether. A fast-moving yellow band and a slower-moving gray band were discarded and the very strong orange band with yellow-green fluorescence was then collected. The solvent was evaporated and the residue dissolved in 100 cc. of heptane containing 1 cc. of triethylamine. The solution was evaporated to 25 cc. and chilled in Dry Ice to precipitate the product, sintering at 110°, m.p. 165°.

2-Nitro-4-diethylaminoacetanilide.[9]—A solution of 20.6 g. (0.1 mole) of 4-diethylaminoacetanilide in 100 cc. of concentrated sulfuric acid was cooled to −10° and a solution of 6.9 g. (0.1 mole) of fuming nitric acid (d. 1.43) in 50 cc. of concentrated sulfuric acid at the same temperature was added in four portions at five-minute intervals. The mixture was kept at −5° until there was no temperature rise when placed in an ice-bath. This required one and three-quarter hours. The reaction mixture was added to 600 g. of ice and the resulting solution was brought to *p*H 2–4 by the addition of 390 cc. of concentrated aqueous ammonia. A black tar that crystallized to a red solid separated rapidly. This was crystallized from 600 cc. of heptane using 2 g. of Darco; yield 13 g. (52%); m.p. 89–90°. Neutralization of the aqueous mother liquors gave 6 g. of starting material.

Anal. Calcd. for $C_{12}H_{17}N_3O_3$: N, 16.7. Found: N, 16.8.

The compound forms a picrate, m.p. 154–156°, from aqueous solution and a hydrochloride from alcoholic hydrochloric acid, m.p. 175–180°.

2-Nitro-4-diethylaminoaniline Hydrochloride.—Twelve and six-tenths grams (0.05 mole) of 2-nitro-4-diethylaminoacetanilide was mixed with 25 cc. of 20% hydrogen chloride in alcohol. Heat was evolved and the mixture solidified. The mixture was refluxed for three hours after the addition of 5 cc. of water and 5 cc. more of 20% hydrogen chloride in alcohol. The resulting solution was clarified with 2 g. of Darco and one volume of ether was added. The precipitated hydrochloride was collected and dried over phosphorus pentoxide.

Anal. Calcd. for $C_{10}H_{15}N_3O_2 \cdot HCl$: C, 48.8; H, 6.5; N, 17.1. Found: C, 48.7; H, 6.6; N, 17.2.

9-Diethylamino-5-phenyliminobenzo[a]phenazine and 10-Diethylamino-5-phenyliminobenzo[a]phenazine.—Ten and four-tenths grams (0.043 mole) of 2-nitro-4-diethylaminoaniline hydrochloride was suspended in 20 cc. of 95% aqueous methanol and reduced at 35 p.s.i. using 100 mg. of platinum oxide as catalyst. The reduction proceeded rapidly and the solution was filtered into a hydrogen-filled flask containing 11.8 g. (0.043 mole) of 4-anilino-1,2-naphthoquinone,[10] 200 cc. of *n*-butanol, and 3.5 g. (0.043 mole) of anhydrous sodium acetate. The solution was refluxed for two hours under hydrogen, cooled and filtered; yield 13 g. of red brown crystals.

This solid was extracted with one 200-cc. portion and two 100-cc. portions of 1:1 aqueous ammonia to remove unreacted 4-anilino-1,2-naphthoquinone (acidification precipitated 1.1 g.). The ammonia-insoluble portion was dried and extracted with hot heptane to remove the soluble 9-isomer. Cooling of the extracts precipitated any 10-isomer that was dissolved, and this was combined with the heptane-insoluble portion and crystallized twice from *n*-butanol (3.5 g./100 cc.); yield 7.6 g. (45%); m.p. 215–217°.

Anal. Calcd. for $C_{26}H_{24}N_4$: C, 79.6; H, 6.1; N, 14.3. Found: C, 79.8; H, 6.3; N, 14.1.

Evaporation of the original butanol reaction mother liquor gave 7 g. of impure 9-isomer. This was dissolved in 1:1 benzene–ethylene chloride and treated with 10-g. portions of alumina until a strong fluorescence that had been masked by the impurities appeared. The filtered solution was evaporated and combined with the heptane extract of the 10-isomer. This solid was crystallized several times by solution in chloroform, the addition of about twenty volumes of heptane and chilling in Dry Ice; yield 2.9 g. (17%); m.p. sinters 95°, gum by 125° without flowing. This behavior was not changed from impure to highly purified material. On chromatography on alumina from 1:20 chloroform–heptane the material was homogeneous. This material is identical with the product described above in fluorescence, solubility, color and visible spectra in three solvents.

Solvent	Spectra Maxima	Spectra Minima	Color
90% sulfuric acid	653, 437	512	Green
10% sulfuric acid	568		Purple
4% alcoholic KOH	485		Yellow

Anal. Calcd. for $C_{26}H_{24}N_4$: C, 79.6; H, 6.1; N, 14.3. Found: C, 79.1; H, 6.3; N, 14.0.

Thiosulfuric Acids.—2-Amino-5-dimethyl- and 2-amino-5-diethylaminophenylthiosulfuric acids have been described by Bernsthen[11] and the dipropyl homolog was prepared by the same method.

(6) All m.ps. are corrected. The analyses were carried out in these laboratories under the direction of Dr. J. A. Kuck. The values reported are the average of two determinations not differing by more than 0.3.

(7) Prepared by the method of A. Groll (*Ber.*, **19**, 200 (1886)) for the dimethyl compound.

(8) O. Fischer and E. Hepp, *ibid.*, **20**, 1247 (1887).

(9) Method of H. H. Hodgson and J. H. Crook (*J. Chem. Soc.*, 2977 (1932)), for the dimethyl compound.

(10) M. Böniger, *Ber.*, **27**, 25 (1894).

(11) A. Bernsthen, *Ann.*, **251**, 44, 53 (1889).

A mixture of 33.5 g. (0.174 mole) of 4-amino-di-*n*-propylaniline[12] and 122 g. (0.183 mole) of aluminum sulfate·$18H_2O$ in 440 cc. of water was treated with 62.5 g. (0.396 mole) of sodium thiosulfate in 330 cc. of water and 23.8 g. (0.175 mole) of zinc chloride in 75 cc. of water. To the stirred mixture was added 14.5 g. (0.049 mole) of potassium dichromate in 188 cc. of water dropwise over two hours while cooling in an ice-bath. The stirring was continued for one hour and the solid was collected, washed twice with water and once with 1:1 alcohol–ether. Nineteen and three-tenths grams (36%) of a light purple product was obtained, melting with decomposition from 193°. Five grams was purified by solution in 500 cc. of 95% alcohol, treatment with Darco, and concentration to about 200 cc. Three and fifty-five one-hundredths grams of colorless, finely-divided crystals that melted and decomposed from 208° was obtained.

Anal. Calcd. for $C_{12}H_{20}N_2O_3S_2$: N, 9.2. Found: N, 9.1.

Benzophenothiazines.—The three compounds were prepared and purified similarly. The details of one preparation follow.

5-(*p*-Tolylimino)-9-dipropylaminobenzo[a]phenothiazine.—A mixture of 14.9 g. (0.05 mole) of 2-amino-5-di-*n*-propylaminophenylthiosulfuric acid, 11.4 g. (0.05 mole) of 1-*p*-tolylaminonaphthalene,[13] 4.1 cc. of concentrated hydrochloric acid and 375 cc. of glacial acetic acid was stirred at room temperature while adding 184 cc. of 10% potassium dichromate in water over 30 minutes. The resulting dark blue-green mixture was stirred for one hour at room temperature and four hours on a steam-bath. After cooling, the reaction solution was poured into 600 cc. of concentrated aqueous ammonia and 750 cc. of water with stirring. The precipitate was collected, dried, and extracted with three 300-cc. and two 150-cc. portions of boiling benzene. The cooled benzene solutions were combined and treated with 750 cc. of 10% hydrochloric acid and shaken vigorously to precipitate the insoluble hydrochloride of the product. The dark blue precipitate was collected, washed with 10% hydrochloric acid and benzene, and dissolved in 1 liter of alcohol. The alcoholic solution was made basic by the addition of aqueous ammonia, and water was added to completely precipitate the dye base. Fifteen grams (66%) of a dark purple-red solid, m.p. 145–160°, was obtained. Crystallization of 9.7 g. of this from 970 cc. of heptane using Darco gave 5.27 g. of dark brown, finely-divided needles, m.p. 174–176°. Crystallization of the remainder with slower cooling gave 3.26 g. of green crystals with a metallic luster, m.p. 197–199°. The lower-melting form could be crystallized (by working rapidly) without raising the melting point, but seeding of such a solution with the higher-melting form gave the latter. The total yield was 39%.

Anal. Calcd. for $C_{29}H_{29}N_3S$: C, 77.1; H, 6.5; N, 9.3. Found: C, 77.2; H, 6.7; N, 9.2.

The compound showed maximum absorption at 667 mμ in alcoholic hydrochloric acid and 550 mμ in alcoholic ammonium hydroxide.

9-Dimethylamino-5-phenyliminobenzo[a]phenothiazine.—The compound was prepared in 15% yield in the same manner as the dipropyltolyl homolog. Only one melting point was observed, 244–249°.

Anal. Calcd. for $C_{24}H_{19}N_3S$: C, 75.4; H, 5.3; N, 11.0. Found: C, 75.2; H, 5.2; N, 10.8.

The compound showed absorption maxima at 667 mμ in alcoholic hydrochloric acid and at 530 mμ in alcoholic ammonium hydroxide.

9-Diethylamino-5-phenyliminobenzo[a]phenothiazine.—The compound was prepared in 30% yield by the method used above. Only one melting point was observed, 235–241°.

Anal. Calcd. for $C_{26}H_{23}N_3S$: C, 76.3; H, 5.7; N, 10.3. Found: C, 76.3; H, 5.8; N, 10.1.

The compound showed an absorption maximum at 680 mμ in alcoholic hydrochloric acid. The absorption in base was not measured.

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(12) W. A. Jacobs and M. Heidelberger, *J. Biol. Chem.*, **21**, 114 (1915).

(13) Prepared in 60% yield by the method of H. H. Hodgson and E. Marsden (*J. Soc. Chem. Ind. Trans.*, **58**, 156 (1939)) from *p*-toluidine and 1-naphthylamine using 1 mole % of HI as catalyst.

[CONTRIBUTION FROM THE RESEARCH DIVISION, SHARP AND DOHME, INC., AND THE RESEARCH LABORATORIES, MERCK AND CO., INC.]

Isolation of Crystalline Biocytin from Yeast Extract

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Biocytin has been isolated in crystalline form from yeast extract. The isolation involved adsorption on norit A, elution with aqueous ammonia, adsorption on superfiltrol–celite, elution with aqueous ethanolic ammonia, chromatography on superfiltrol–celite, chromatography on alumina, partition with butanol and cresol, countercurrent distribution and crystallization from water.

The isolation of crystalline biocytin from yeast extract was reported.[2] The present paper describes details of the procedures employed for the isolation of biocytin in crystalline form.

The term biocytin (Gr. Kútos, cell) has been used to designate the predominant form of biotin occurring in many solubilized natural products, especially those originating from the controlled autolysis of actively metabolizing material such as yeast extract.[2,3] Biocytin is characterized microbiologically by its availability as a source of biotin to *Lactobacillus casei*, *Lactobacillus delbrückii* LD5, *Lactobacillus acidophilus*, *Streptococcus fecalis* R, *Neurospora crassa* and *Saccharomyces carlsbergensis* and by its unavailability as a source of biotin to *Lactobacillus arabinosus*, *Lactobacillus pentosus* and *Leuconostoc mesenteroides* P-60. When subjected to strong acid hydrolysis (at least 3 N at 120° for 1 hour) biocytin yields biotin, or its microbiological equivalent, as a moiety.

When any natural product is isolated in pure form for the first time, there is a question as to whether or not the pure substance obtained is identical with the factor as it occurs in natural materials. Consideration of the data obtained during the course of the isolation work furnishes considerable presumptive evidence that the crystalline substance isolated is identical with biocytin as it occurs in yeast extract. Thus, although a number of steps were essential in the isolation procedure so that the

(1) E. I. du Pont de Nemours & Company Inc., Newark, Delaware

(2) L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf and K. Folkers, THIS JOURNAL, **72**, 1048 (1950); L. D. Wright, E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant and K. Folkers, *Science*, **114**, 635 (1951).

(3) L. D. Wright and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.*, **56**, 95 (1944).