N-Substitution in the 2-Amino- and 2,7-Diaminophenoxazin-3-one Ring System. Application to the Preparation of Actinomycin D Analogs

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A method is described for the direct N^2-monoalkylation of the 2-aminophenoxazin-3-one system by treatment with dimethylsulfonium methylide in DMSO-THF, which acts as a base by abstracting a proton from the 2-amino function to form a stabilized anion, which is then alkylated by reaction with an alkyl halide. Selective N^7-monoalkylation of the 2,7-diaminophenoxazin-3-one system can be accomplished by reaction with aromatic aldehydes in glacial acetic acid to give Schiff bases, which are then reduced with dimethylamine borane. These reactions have been applied to the preparation of N^2-benzyl- and 7-benzylaminoactinomycin D.

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The actinomycins are a series of chromophoric antibiotics isolated from *Streptomyces* culture (1). These compounds are composed of a substituted phenoxazinone ring system with two cyclic pentapeptide lactones attached at the 1 and 9 positions, and they differ only in the amino acid composition of the peptides. One of these compounds, actinomycin D (AMD), **1b**, has found limited but effective use in cancer chemotherapy, most notably in the treatment of Wilm's tumor and gestational choriocarcinoma (2). Because of its limited range of effectiveness against tumors, and also because of its narrow therapeutic index and high toxicity, the chemical structure of AMD has been modified in various ways in attempts to improve its clinical usefulness. Modifications have been made both in the phenoxazinone chromophore moiety and in the pentapeptide lactone regions of various actinomycins. This work has been conducted in a number of laboratories, including our own, and several reviews (3-6) are available which describe the chemical and biological properties of these compounds.

For several years we have carried out extensive investigations on the synthesis and biological properties of AMD analogs (7-9) and on their use as fluorescent DNA-binding agents (7,10). Most of this work has been centered around the preparation and antitumor evaluation of 7-substituted and oxazolo- and oxazino-substituted AMD analogs. We showed (8) that 7-nitro and 7-amino AMD are comparable to AMD in DNA-binding specificity and in growth-inhibitory properties against four different transplantable mouse tumors. In view of these results it seemed desirable to evaluate other 7-substituted AMD analogs as antitumor agents.

The 2-amino function of AMD appears to play an important role in the antibacterial activity and DNA-binding properties of the actinomycins (3,4,6). Meienhofer, Johnson, and colleagues have recently shown (11) that 2-deamino AMD was inactive against the L1210 mouse leukemia and virtually inactive against the P388 mouse leukemia. However, they also reported that N^2-(γ-hydroxypropyl)AMD has activity comparable to AMD against these two mouse leukemias. Thus, it appeared that certain other N^2-substituted AMD analogs might be worth investigating as antitumor agents.

In this paper we wish to report a method for the direct N^2-alkylation of the 2-aminophenoxazin-3-one ring system and methods for selective N^7-substitution of the 2,7-diaminophenoxazin-3-one ring system. Initially, these reactions were performed on the model chromophore compounds **1a** and **8a**, which were previously synthesized in this laboratory (8). However, we will also describe here the preparation of two AMD analogs using these procedures.

Many N^2-substituted analogs of the actinomycins, (6,11) and of model chromophore systems, (12,13) have been prepared. One synthetic method commonly used by investigators is to convert the 2-amino group into a hydroxyl group by acid hydrolysis, followed by treatment of the hydroxy compound with thionyl chloride to give the 2-chloro substituent. Treatment of the latter with various amines then leads to N^2-monosubstituted or disubstituted compounds. The value of our improved methodology for one-step conversion of AMD into N^2-substituted derivatives is apparent when one considers the three-step method now employed and the high cost of AMD as a starting material. Another method, which was reported by Brockmann for the preparation of N^2-monosubstituted actinomycins, (14) consists of the reductive alkylation of the amino group with hydrogen in the presence of certain ketones, and then air oxidation of the reduced ring system to regenerate the phenoxazinone, but no experimental details are available. To our knowledge, aldehydes have not been reported to undergo a similar reductive alkylation with 2-aminophenoxazin-3-ones, possibly because they react to form oxazolophenoxazines (15).

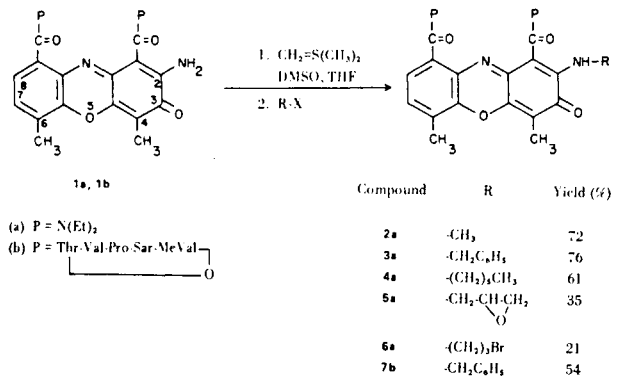
We became interested in determining whether the 2-amino group of **1a** could be directly alkylated with alkyl halides under appropriate conditions. In this ring system, the 2-amino group is actually a vinylogous amide and is therefore only weakly basic. We thought that if **1a** were

treated with a very strong base, proton abstraction might occur at the 2-amino function, thus generating an anion which would be stabilized by the highly conjugated ring system. Addition of an alkyl halide should than lead to the N^2-alkylated derivative.

When **1a** was added to a solution of lithium methylsulfinyl carbanion in DMSO (prepared by adding *n*-butyllithium to dry DMSO) (16) under nitrogen at room temperature, the dark red color of **1a** immediately changed to a deep blue-green. Addition of excess methyl iodide then led to the discharge of the blue-green color after about 15 minutes and regeneration of the red color. Thin layer chromatography (silica gel: 10% methanol-ethyl acetate) indicated that the starting material had been consumed, and that a new product, moving slightly faster than **1a**, had been formed along with minor amounts of other products. Processing of the reaction mixture resulted in the isolation of the major product, which was identified

Scheme I

N^2-Alkylation of the 2-Aminophenoxazin-3-one Ring System



Compound	R	Yield (%)
2a	$-CH_3$	72
3a	$-CH_2C_6H_5$	76
4a	$-(CH_2)_3CH_3$	61
5a	$-CH_2$-CH-CH_2 (O)	35
6a	$-(CH_2)_3Br$	21
7b	$-CH_2C_6H_5$	54

as the N^2-methylated compound **2a** (Scheme I). Subsequently, it was found that if the weaker base, dimethylsulfonium methylide, (17) was used instead of the methylsulfinyl carbanion, the reaction proceeded more cleanly to give **2a**, with fewer by-products. *n*-Butyllithium was added to a solution of trimethylsulfonium iodine in DMSO-THF, at 0° under nitrogen, to generate the ylide, followed by the addition of **1a** in DMSO. After several minutes, methyl iodide was added to the blue-green solution, which was then allowed to stir at room temperature until the color changed to red. In this reaction, the nucleophilic sulfur ylide, rather than acting as a methylene-transfer agent (17), abstracts a proton from the 2-amino function of **1a**.

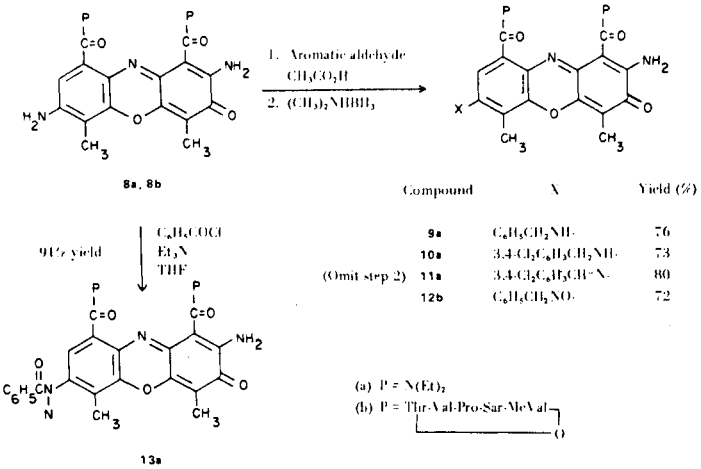
The structure of **2a** was confirmed by elemental analysis and by nmr spectroscopy. The 2-amino hydrogens of **1a**, which appear as a single peak at δ 5.44 (deuteriochloroform; TMS internal standard) are clearly absent in the spectrum of **2a**, and a new broad peak (NH) appears at δ 6.34. The *N*-methyl group of **2a** appears as a doublet centered at δ 3.08, overlapping with the methylene hydrogens of the two diethylamide groups.

In similar reactions, benzyl bromide reacted rapidly at room temperature with the anion of **1a** to give **3a**. 1-Bromohexane reacted only slowly at room temperature to give **4a**, but at 60° the reaction proceeded more rapidly to completion (~45 minutes). Epibromohydrin and 1,3-dibromopropane were used to give **5a** and **6a** respectively, but in these reactions the yields of products were low because of extensive dehydrohalogenation, which generated much starting material. However, the alkylated products could easily be separated from **1a** by column chromatography on silica gel. When AMD, **1b**, was treated with the sulfur ylide under the same conditions, the color of the anion generated was deep purple. Alkylation with a small excess of benzyl bromide proceeded much more slowly than in the case of **1a**, possibly because of the greater steric hindrance at the 2-amino group in **1b**. However, with a large excess of benzyl bromide, **7b** was formed in good yield at room temperature.

We next turned our attention toward methods for selective substitution at the more basic 7-amino function of the diamino compound **8a** (8) (Scheme II). It was

Scheme II

N^7-Substitution of the 2,7-Diaminophenoxazin-3-one Ring System



Compound	X	Yield (%)
9a	$C_6H_5CH_2NH$-	76
10a	3,4-$Cl_2C_6H_3CH_2NH$-	73
(Omit step 2) 11a	3,4-$Cl_2C_6H_3CH$=N-	80
12b	$C_6H_5CH_2NO$-	72

found that **8a** reacted with aromatic aldehydes only at the 7-amino group, at room temperature in glacial acetic acid, to give imines, which, without isolation, could be rapidly reduced to monoalkylated derivatives by the addition of dimethylamine borane (18). Thus, benzaldehyde and 3,4-dichlorobenzaldehyde gave **9a** and **10a** respectively. The imine **11a** was isolated by omitting the reduction step, and was found to be a stable compound. 7-Amino AMD, (8) **8b**, reacted cleanly with benzaldehyde and dimethylamine borane to give the 7-benzylamino derivative **12b**. Aliphatic aldehydes have also been found to react with **8a** under these conditions, but the yields of 7-alkylated products are low and much starting material is recovered unchanged. This may be due to an unfavorable equilibrium in the formation of the intermediate imine. It is possible that certain catalysts, and/or other solvents, may be found to

Table I

Analytical Data

Compound No.	Molecular Formula	Analyses			
2a	$C_{25}H_{32}N_4O_4$	Calcd: C, 66.35;	H, 7.13;	N, 12.38	
		Found: 66.30	7.10	12.32	
3a	$C_{31}H_{36}N_4O_4$	Calcd: C, 70.43;	H, 6.86;	N, 10.60	
		Found: 70.38	6.90	10.69	
4a	$C_{30}H_{42}N_4O_4$	Calcd: C, 68.93;	H, 8.10;	N, 10.72	
		Found: 68.80	8.02	10.80	
5a	$C_{27}H_{34}N_4O_5$	Calcd: C, 65.57;	H, 6.93;	N, 11.33	
		Found: 65.49	6.96	11.22	
6a	$C_{27}H_{35}BrN_4O_4$	Calcd: C, 57.96;	H, 6.30;	N, 10.01;	Br, 14.28
		Found: 57.93	6.29	9.99	14.10
7b	$C_{69}H_{92}N_{12}O_{16}\cdot 2H_2O$	Calcd: C, 59.98;	H, 7.00;	N, 12.17	
		Found: 60.03	6.97	12.03	
9a	$C_{31}H_{37}N_5O_4$	Calcd: C, 68.48;	H, 6.86;	N, 12.88	
		Found: 68.19	6.91	12.77	
10a	$C_{31}H_{35}Cl_2N_5O_4$	Calcd: C, 60.78;	H, 5.76;	N, 11.43;	Cl, 11.58
		Found: 60.54	5.85	11.29	11.39
11a	$C_{31}H_{33}Cl_2N_5O_4\cdot H_2O$	Calcd: C, 59.23;	H, 5.61;	N, 11.14;	Cl, 11.28
		Found: 59.35	5.69	11.07	11.37
12b	$C_{69}H_{93}O_{16}\cdot 2H_2O$	Calcd: C, 59.34;	H, 7.00;	N, 13.04	
		Found: 59.26	6.92	12.89	
13a	$C_{31}H_{35}N_5O_5$	Calcd: C, 66.77;	H, 6.33;	N, 12.56	
		Found: 66.50	6.21	12.41	

improve the yields in these reactions. Benzoylation of **8a**, even in the presnece of excess benzoyl chloride, gave **13a** in excellent yield.

These reactions are being used to prepare additional AMD analogs. The biological properties of **7b**, **12b**, and additional AMD analogs, both *in vitro* and *in vivo*, will be determined when sufficient material is available and will be reported subsequently.

EXPERIMENTAL

Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatud (Laboratory Devices, Inc., Cambridge, Mass.) and are uncorrected. Ir spectra were taken with a Perkin-Elmer Model 137B double-beam spectrophotometer, and uv spectra with Cary Model 11 and 15 spectrophotometers. Nmr spectra were performed on a Varian T60-A spectrometer in deuteriochloroform with tetramethylsilane as internal standard, unless otherwise indicated. Tlc's were done on Eastman silica gel Chromogram sheets with 10% methanol-ethyl acetate as developing solvent. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn.

General Method for the N^2-Alkylation of 2-Amino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-3*H*-phenoxazin-3-one (**1a**).

To a stirred solution of trimethylsulfonium iodide (1.25 mmoles) in dry DMSO (5 ml.) and THF (7 ml.), at 0° under nitrogen, was added a solution of *n*-butyllithium in hexane (1.2 mmoles, Alfa, Beverly, Mass.). After 3 minutes a solution of **1a** (1.0 mmole) in DMSO (7 ml.) was added, and then after another 3 minutes the alkyl halide (4 mmoles for **2a-5a**; 20 mmoles for **6a**) was added to the deep blue-green solution. The reaction mixture was then allowed to warm to room temperature, or heated slightly, until the color changed to red. The reactions can be followed by tlc, the products **2a-6a** moving faster than the starting material **1a**. Reaction times and temperatures were as follows: For compounds **2a** and **3a** (20-30 minutes, room temperature), **4a** (1 hour, 60°), **5a** (1.5 hours, 60°), **6a** (2 hours, room temperature). The reaction mixture was then poured into a mixture of water (50 ml.) and saturated aqueous salt solution (50 ml.) and extracted with chloroform (4 x 40 ml.). The combined extracts were washed with water (3 x 50 ml.), saturated aqueous salt solution (50 ml.), dried (sodium sulfate), and evaporated. Compounds **2a-4a** were recrystallized directly from ethyl acetate-hexane, while **5a** and **6a** had to be chromatographed on silica gel (10% methanol-ethyl acetate) to separate them from starting material, and then recrystallized. Pertinent physical data for these compounds are listed below.

Compound **1a**.

This compound had m.p. 198-199°; nmr: δ 2.24 (s, 4-CH_3), 2.52 (s, 6-CH_3), 5.44 (s, 2-NH_2), 7.12 (d, 7-H), 7.30 (d, 8-H).

Compound **2a**.

This compound had m.p. 238-239°; nmr: δ 2.20 (s, 4-CH_3), 2.50 (s, 6-CH_3), 3.08 (d, 2-NCH_3), 6.34 (broad, 2-NH).

Compound **3a**.

This compound had double m.p. 115-120° and 184-186°; nmr: δ 4.60 (m, 2-N-CH_2), 6.43 (broad, 2-NH), 7.34 (s, C_6H_5).

Compound **4a**.

This compound had m.p. 150-152°.

Compound **5a**.

This compound had m.p. 168-171°; nmr: δ 3.4-4.1 (m, 2-N-CH_2 and CH-O overlapping with the methylene hydrogens of

the diethylamides - proven by integration), the -CH_2O- hydrogens are in the region of δ 2.1-2.8, under the 4- and 6- methyls; 6.43 (broad, 2-NH).

Compound **6a**.

This compound had m.p. 155-156°; nmr: δ 2.30 (m, C-CH_2-C), 3.1-4.0 (m, 2-N-CH_2 and -CH_2Br overlapping with the methylene hydrogens of the diethylamides), 6.20 (broad, 2-NH).

N^2-Benzyl AMD (**1b**).

To a stirred solution of trimethylsulfonium iodide (45 mg., 0.22 mmole) in dry DMSO (5 ml.) and THF (8 ml.), under nitrogen at 0°, was added *n*-butyllithium in hexane (0.2 mmole). After 3 minutes a solution of AMD (125 mg., 0.1 mmole) in DMSO (7 ml.) was added, and after another 3 minutes benzyl bromide (1.71 g., 10 mmoles) was added to the deep purple solution. Stirring was continued at room temperature for 1 hour (after 30 minutes the solution changed to red) and the reaction mixture was then processed as described above. The crude product was chromatographed on silica gel (5% methanol-ethyl acetate) and then recrystallized from ethyl acetate-hexane to give an orange solid (75 mg., 54%), m.p. 235-240° dec. Tlc on silica gel (isobutyl alcohol:formic acid:water, 75:13:12) showed a single spot at R_f 0.71, while AMD had R_f 0.76. The nmr spectrum (acetone-d_6, TMS) was similar to AMD except that phenyl multiplets could be seen in the region of δ 7.05-7.2 which were absent in the spectrum of AMD. The ir spectrum showed lactone carbonyl absorption at 5.72 μ.

General Method for the N^7-Alkylation of 2,7-Diamino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-3*H*-phenoxazin-3-one (**8a**).

A solution of **8a** (0.2 mmole) and the aromatic aldehyde (0.6 mmole) in glacial acetic acid (5 ml.) was stirred at room temperature for 1 hour, followed by the addition of dimethylamine borane (0.6 mmole) in one protion. After 30 minutes the solution was poured into chloroform (50 ml.), extracted with saturated aqueous sodium bicarbonate (2 x 50 ml.), and the chloroform solution was then dried (sodium sulfate) and evaporated. The crude products were recrystallized from ethyl acetate-hexane. Compound **11a** was obtained simply by eliminating the dimethylamine borane reduction step. Pertinent physical data for these compounds are listed below.

Compound **9a**.

This compound had m.p. 140-150°; nmr: δ 2.25 (s, 4-CH_3), 2.32 (s, 6-CH_3), 4.52 (m, 7-NH and N-CH_2), 5.17 (s, 2-NH_2), 6.59 (s, 8-H), 7.34 (s, C_6H_5).

Compound **10a**.

This compound had m.p. 275-277°; nmr: δ 4.45 (m, 7-NCH_2), 4.74 (m, 7-NH), 5.19 (s, 2-NH_2), 6.45 (s, 8-H), 7.05-7.45 (m, $C_6H_3Cl_2$).

Compound **11a**.

This compound had m.p. 265-267°; nmr: δ 5.42 (s, 2-NH_2), 6.90 (s, 8-H), 7.60 and 8.00 (m, $C_6H_3Cl_2$), 8.38 (s, CH=N).

7-Benzylamino AMD (**12b**).

A solution of **8b** (51 mg., 0.04 mmole) and benzaldehyde (42 mg., 0.4 mmole) in glacial acetic acid (4 ml.), with 100 mg. of molecular sieves (4Å) added, was stirred at room temperature for 30 minutes, followed by the addition of dimethylamine borane (36 mg., 0.6 mmole). After 15 minutes, tlc showed the presence of some unreacted **8b**, R_f 0.59, and a faster moving spot, R_f 0.71. Therefore, an additional quantity of benzaldehyde (0.4 mmole) was added again, followed after 15 minutes by dimethylamine borane (0.6 mmole). The reaction mixture was stirred for 30 minutes and then processed as described above. The product was recrystallized from carbon tetrachloride to yield violet crystals (40 mg., 72%), m.p. 240-250° dec. The ir spectrum showed a peak at 5.7 μ indicating lactone ring carbonyl absorption.

2-Amino-7-benzoylamino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-3*H*-phenoxazin-3-one (**13a**).

To a stirred solution of **8a** (91 mg., 0.2 mmole) and triethylamine (61 mg., 0.6 mmole) in THF (5 ml.) at room temperature was added benzoyl chloride (62 mg., 0.44 mmole). After stirring for 90 minutes, chloroform (50 ml.) was added and the mixture was then extracted with aqueous sodium bicarbonate (5%), dried (sodium sulfate), and evaporated. The residue was recrystallized from ethyl acetate-hexane to give an orange solid (102 mg., 91%), double m.p. 155-170° and 264-268°; nmr: δ 1.92 (s, 4-CH_3), 2.18 (s, 6-CH_3), 5.60 (s, 2-NH_2), 7.30 (s, 8-H), 7.56 and 8.12 (m, C_6H_5CO-), 9.88 (s, -CONH).

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