[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF CINCINNATI]

PEPTIDE DERIVATIVES OF THE CARCINOGEN 2-AMINOFLUORENE

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The versatility and importance of 2-aminofluorene and some of its derivatives has been well recognized in the field of oncology because of the ability of these compounds to promote tumor formation in a variety of sites. The original report of Wilson, De Eds, and Cox (1) in this field has been followed by much work, particularly by F. Bielschowsky, J. H. and E. K. Weisburger, H. P. Morris, and F. E. Ray. In an effort to achieve a greater understanding of this phenomenon, a group of amino acid and peptide derivatives of 2-aminofluorene has been synthesized. Because of the intimate relationship which may exist between carcinogenesis and proteins or protein precursors, it seemed possible that some valuable information might be forthcoming from a study of the carcinogenic activity of these new derivatives.[2]

All but one of the compounds described herein were prepared by coupling phthalyl protected amino acids (2) to either 2-aminofluorene or 2-amino-7-acetamidofluorene. The protective group was removed with hydrazine hydrate to produce the free amine. Repetition of the coupling procedure with the new amine led to the formation of a dipeptide derivative of either the 2-amino- or the 2,7-diamino-fluorene.

Although the method employed has generally proved useful for peptide syntheses, considerable difficulty was encountered in two phases of this work, namely the selection of a suitable solvent for some of the intermediates containing the fluorene nucleus and the separation of the free amine from phthalhydrazide subsequent to hydrazinolysis. The former difficulty was circumvented by the use of dimethylformamide as a solvent, while the troublesome separation was overcome by employing cold aqueous sodium hydroxide to remove the phthalhydrazide as its soluble sodium salt. Separation *via* the amine hydrochloride was unsatisfactory because of the low solubilities of the hydrochlorides. Several reports in the literature (3, 4) refer to the use of various bases for the separation of the products, and Mosher (5) describes a method utilizing potassium hydroxide.

A summary of some of the compounds prepared in this investigation is given in Tables II and III.

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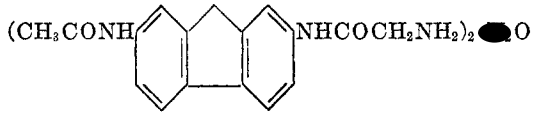
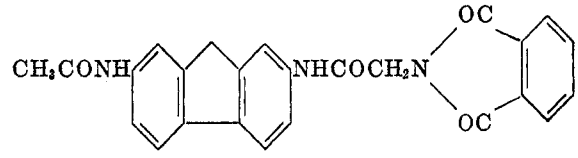
[2] Biological testing of the compounds has been undertaken by Dr. F. E. Ray, Director, Cancer Institute, University of Florida, Gainesville, Florida. Because of the length of time required for testing, no results are yet available.

An attempt was made to synthesize 2-glycylamino-7-acetamidofluorene, but the data indicated that a monohydrated dimer (IX) was formed after hydrazinolysis of the phthalyl derivative (X). The analyses substantiated this fact



IX



X

although the melting points and analytical results varied slightly with the solvent used for recrystallization. For example, from chlorobenzene there was obtained a sample which had a lower melting point and somewhat higher percentages of carbon and nitrogen than a sample which was recrystallized from ethanol. This was in conformity with expectations, since the monohydrated dimer would tend to dissociate in a more polar solvent; the calculated carbon and nitrogen values were higher for the monomer.

Evaporating the filtrates from the ethanol recrystallizations yielded a substance whose melting point agreed with that of the sample obtained from chlorobenzene, indicating a higher solubility of this material. The ebullioscopic molecular weight of the monohydrated dimer in chlorobenzene was 578, within experimental error of the calculated value of 608. The molecular weight of the same substance was 387 in the more polar acetone, evidently a mixture of monomer and monohydrated dimer, since the theoretical molecular weight of the monomer was 295.

A sample of the monohydrated dimer was dried to constant weight at 80° under a vacuum to duplicate the procedure used for the analyses. A known quantity of the dried sample was heated at 120° under a vacuum and weighed at intervals. The curve for the per cent weight loss with time exhibited a gradual rise, leveled at 3%, and then increased sharply as decomposition became apparent. The calculated weight loss for the monohydrated dimer was 2.96%. The melting point of the sample used was 205–206° before drying but 194–195° after drying under these conditions.

Recrystallization of 2-glycylaminofluorene (V) and 2-(di-DL-phenylalanyl)-aminofluorene (VIII) in various solvents also afforded samples which differed in melting points, indicating the possibility of a similar phenomenon.

Initially, an attempt was made to synthesize 2-glycylaminofluorene by an alternate route. Chloroacetyl chloride was reacted with 2-aminofluorene to produce 2-chloroacetamidofluorene (6). Despite variations in the conditions the

principal product from the amination of the chloro compound was the secondary amine α-amino-bis(2-acetamidofluorene) as determined from qualitative tests and analytical data.

EXPERIMENTAL

Phthalyglycine was prepared according to the procedure of Drechsel (9) with a slight modification. Phthalic anhydride (using a slight excess) was fused in a flask preheated in an oil-bath to 190–200°. In this way any sublimation loss was eliminated and glycine could be added immediately in small portions to the stirred melt. The yield obtained was 98%; m.p. 194–198°.

Phthalyl-DL-phenylalanyl chloride. Phthalyl-DL-phenylalanine (2) (8.72 g., 0.03 mole) and thionyl chloride (10 ml., 0.134 mole) were heated to reflux temperature. The material dissolved within 30 minutes and after another 30 minutes of heating ligroin (40–60°) (90 ml.) was added to precipitate the product. The phthalyl-DL-phenylalanyl chloride was filtered, washed with ligroin and dried (9.07 g., 98%); m.p. 123–125°.

Phthalylaminoacylaminofluorenes (I–IV). These compounds were prepared by the same general methods. The details of the preparation of 2-phthalyglycylaminofluorene (I) may be considered as typical of all the others. Variations in reactants, concentrations, reaction times, solvents, and other details are given in Table IA. The physical constants and analyses are reported in Table II.

2-Phthalylglycylaminofluorene (I). A one-liter, three-necked round-bottom flask provided with a dropping funnel, rubber-sealed stirrer, and calcium chloride filled drying tube

TABLE I

DETAILS FOR THE PREPARATION OF COMPOUNDS

A. PHTHALYLAMINOACYLAMINOFLUORENES

NO.	ACYL HALIDE		AMINE		PYRIDINE (Moles)	BENZENE (ML.)	TIME (Hrs)	RECRYST. SOLVENT
	Acyl Chloride	Moles	Amine	Moles				
II	Phthalylglycyl	0.072	2-Glycylamino fluorene	0.072	0.021	600	2	Dimethyl formamide and ethanol
III	Phthalyl-DL-phenylalanyl	.029	2-Aminofluorene	.028	.084	570	3	Same as II
IV	Phthalyl-DL-phenylalanyl	.004	2-DL-Phenylalanyl-aminofluorene	.004	.012	135	2.5	Ethanol

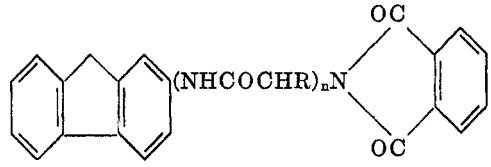
B. AMINOACYLAMINOFLUORENES

NO.	PHTHALIMIDE		HYDRAZINE HYDRATE (Moles)	VOLUME OF ETHANOL (ML.)	TIME (Hrs)	RECRYST. SOLVENT	YIELD, %
	Phthalimide	Moles					
VI	Diglycylamino fluorene	0.002	0.004[a]	300	2	Chlorobenzene	85
VII	DL-Phenylalanyl amino-fluorene	.003	.006[a]	325	1.5	Chlorobenzene[c]	99
VIII	Di-DL-phenylalanyl aminofluorene	.0014	.0084[b]	250	8	Ethanol	99

[a] Added in two equal portions one hour apart.
[b] Added in three equal portions one hour apart.
[c] Under a nitrogen atmosphere.

TABLE II

PHTHALYLAMINOACYLAMINOFLUORENES



No.	R	n	M.P.,[d] °C.	FORMULA	ANALYSIS					
					C		H		N	
					Calc'd	Found	Calc'd	Found	Calc'd	Found
I[e]	H	1	302.0–302.5	$C_{23}H_{16}N_2O_3$	77.14	76.98	3.99	4.03	6.93	6.90
II	H	2	301.0–302.0	$C_{25}H_{19}N_3O_4$	70.58	70.46	4.50	4.66	9.88	9.72
III	$C_6H_5CH_2$	1	229.5–230.0	$C_{30}H_{22}N_2O_3$	78.58	78.78	4.84	4.81	6.11	5.99
IV	$C_6H_5CH_2$	2	234.5–235.5	$C_{39}H_{31}N_3O_4$	77.34	77.59	5.16	5.06	6.94	6.69

[d,e] For footnotes *cf.* Table III.

was placed into an ice bath (5°). Dry benzene (400 ml.), 2-aminofluorene[3] (3.7 g., 0.02 mole) and pyridine (1.6 g., 0.06 mole) were introduced. Phthalylglycyl chloride (2, 10) (4.5 g., 0.02 mole) dissolved in 80 ml. of dry benzene was added dropwise with stirring in about 20 minutes. After one hour the ice-bath was removed and stirring was continued for an additional hour. The mixture was filtered and the solid product was washed successively with benzene, alcohol, water, and alcohol. The dried 2-phthalylglycylaminofluorene weighed 7 g. (95%); m.p. 302–302.5° after recrystallization from dimethylformamide-ethanol.

Aminoacylaminofluorenes (V–VIII). Since these compounds were all prepared by the same general procedure, the details of the preparation of 2-glycylaminofluorene (V) will serve to illustrate the method. Variations in molar quantities of reactants, time of reaction, amount of solvent, and solvent used for recrystallization are given in Table IB. The physical constants, yields, and analyses are given in Table III.

2-Glycylaminofluorene (V). A solution containing 2-phthalylglycylaminofluorene (3.5 g., 0.0095 mole) and hydrazine hydrate (1 g., 0.02 mole) in 500 ml. of 95% ethanol was refluxed until clear, usually 1.5–2 hours. The solvent was removed by vacuum-distillation, and the solid, after being washed with a small quantity of water to remove the excess hydrazine, was filtered and dried. Combined portions (33 g.) from several runs were suspended in a cold solution of 225 g. of sodium hydroxide in 525 ml. of water and stirred vigorously for ten hours at a temperature of 10–15°. After diluting with water to four times the original volume, the suspension was filtered; the product was washed with water until all the sodium hydroxide was removed, and dried. The yield of 2-glycylaminofluorene, calculated from 2-phthalylglycylaminofluorene, was quantitative; m.p. 183–184° after recrystallization from chlorobenzene.

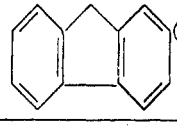
2-Phthalylglycylamino-7-acetamidofluorene (X).[4] A suspension of 2-amino-7-acetamido-

[3] 2-Aminofluorene, prepared by the method of Sampey and Reid (7), was obtained in 93% yield when the powdered zinc and 2-nitrofluorene (8) were intimately mixed prior to and during the reduction procedure. Quantitative reduction of larger amounts of 2-nitrofluorene was obtained using an Aminco high pressure hydrogenator with alcohol as a solvent and a pressure of 100 p.s.i. at 80°.

[4] Where similar reactions were performed, the same type of equipment was utilized for all, but is described only in the initial procedure.

TABLE III

AMINOACYLAMINOFLUORENES



No.	R	n	M.P.,[d] °C.	FORMULA	ANALYSIS					
					C		H		N	
					Calc'd	Found	Calc'd	Found	Calc'd	Found
V	H	1	183.0–184.0	$C_{15}H_{14}N_2O$	75.61	75.61	5.92	5.82	11.76	11.70
VI	H	2	187.0–188.0	$C_{17}H_{17}N_3O_2$	69.13	69.39	5.80	5.84	14.23	13.93
VII	$C_6H_5CH_2$	1	160.0–160.5	$C_{22}H_{20}N_2O$	80.46	80.50	6.14	6.26	8.53	8.49
VIII	$C_6H_5CH_2$	2	185.5–186.0	$C_{31}H_{29}N_3O_2$	78.29	78.55	6.15	5.96	8.84	8.61

[d] All melting points were taken with open capillary tubes in a 2.5″ × 3.5″ cylindrical iron block at a depth of 76 mm. using a calibrated 76-mm. immersion thermometer. [e] This compound has been reported by Yingst in a University of Cincinnati M.S. thesis (unpublished). His yield was 70% less and the melting point, seven degrees lower than those obtained by the procedure described herein. Complete analysis was not reported.

fluorene (8) (6.6 g., 0.03 mole) in 300 ml. of dry benzene containing pyridine (6.6 g., 0.08 mole) was cooled to 5°. A solution of phthalylglycyl chloride (6.2 g., 0.03 mole) in 200 ml. of dry benzene was added dropwise with stirring in thirty minutes. After stirring an additional hour the mixture was filtered; the cake was washed successively with benzene, alcohol, water and alcohol. The dried 2-phthalylglycylamino-7-acetamidofluorene weighed 8.3 g. (70.5%); m.p. 344.5–345.5° after recrystallization from dimethylformamide-ethanol.

Anal. Calc'd for $C_{25}H_{19}N_3O_4$: C, 70.58; H, 4.50; N, 9.88.
Found: C, 70.38; H, 4.38; N, 9.60.

Monohydrated dimer of 2-glycylamino-7-acetamidofluorene (IX). A solution containing 2-phthalylglycylamino-7-acetamidofluorene (2.92 g., 0.007 mole) and hydrazine hydrate (0.34 g., 0.007 mole) in 600 ml. of 95% ethanol was refluxed. After 2.25 hours a similar quantity of hydrazine hydrate in 50 ml. of 95% ethanol was added; this was repeated twice more within the next 7.75 hours. Refluxing was continued another two hours and the clear solution was vacuum-distilled to remove the solvent. The solid weighing 3.15 g. was stirred efficiently in 300 ml. of 28% sodium hydroxide for 7.75 hours at a temperature which rose from 5° to room temperature during that time. After dilution to four times the original volume with water, the suspension was filtered and the solid residue was washed to remove the alkali. The dried monohydrated dimer of 2-glycylamino-7-acetamidofluorene was obtained in quantitative yield; m.p. 189–192°, from chlorobenzene; m.p. 210–211°, from ethanol.

Anal. Calc'd for $(C_{17}H_{17}N_3O_2)_2 \cdot H_2O$: C, 67.09; H, 5.96; N, 13.81.
Found: m.p. 189–192°: C, 67.48; H, 5.83; N, 13.74.
C, 67.46; H, 5.86; N, 13.45.
C, 67.46; H, 5.71; N, 13.68.
m.p. 210–211°: C, 67.29; H, 6.25; N, 13.59.
C, 67.15; H, 6.06.
Mol. Wt. Det'n. Calc'd for $(C_{17}H_{17}N_3O_2)_2 \cdot H_2O$: 608.
Calc'd for $C_{17}H_{17}N_3O_2$: 295.
Found (ebullioscopic): Chlorobenzene, 578; Acetone, 387.

α-Amino-bis(2-acetamidofluorene). Approximately 20% ammoniacal ethanol solutions were prepared by bubbling gaseous ammonia through ethanol. Such a solution (330 g.) containing ammonia (17 g., 1 mole) and 2-chloroacetamidofluorene (6) (5 g., 0.019 mole)

was placed in the one-liter glass liner of an Aminco high pressure hydrogenator equipped with plugs instead of gas inlet tubes. The solution could also be divided equally among five, 250-ml. glass pressure bottles (caution). After being heated for four hours at 100°, the solution was cooled and vacuum-distilled to remove the solvent. The white α-amino-bis(2-acetamidofluorene) was stirred in water, filtered and dried (5.3 g., 60.5%); m.p. 278.5–279.5° after recrystallization from dimethylformamide.

Anal. Calc'd for $C_{30}H_{25}N_3O_2$: C, 78.41; H, 5.48; N, 9.15.
Found: C, 78.69; H, 5.60; N, 9.29.

SUMMARY

In order to provide some additional derivatives of 2-aminofluorene for carcinogenic studies, the following new compounds were prepared: 2-phthalylamino acid acylaminofluorenes and 2-amino acid acylaminofluorenes; the 7-acetamido derivative of two of the above; α-amino-bis(2-acetamidofluorene).

New or improved methods of synthesis were developed for the intermediates as well as the new compounds. Evidence was given for the existence of a monohydrated dimer of 2-glycylamino-7-acetamidofluorene.

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