gen groups was introduced into bovine serum albumin than into horse serum albumin. Increases both in the ratio of isocyanate to protein and in the percentage of dioxane in the reaction mixture caused the expected increase in the extent of conjugation. Paralleling previous experience with the polycyclic aromatic hydrocarbons,[7,8] the more dioxane-soluble 2'-methyl-4-dimethylaminostilbenyl-4'-isocyanate reacted more completely with the proteins than the less soluble 4-dimethylaminostilbenyl-4'-isocyanate. The greater reactivity of both stilbenyl isocyanates compared with those of the polycyclic aromatic hydrocarbons[7] led to a more complete reaction with the proteins. Preparations Nos. 9 and 10 contained 55 and 62 groups, respectively, whereas the greatest number of hydrocarbon groups introduced into the serum albumins was 38 by the reaction with 1,2-benzanthryl-10-isocyanate under equally favorable experimental conditions.

Horse and bovine serum albumin conjugates containing both high and low numbers of the two stilbenyl prosthetic groups were required for the immunological studies.[12] The blue fluorescence exhibited by solutions of these conjugates was applied advantageously in the serological work.

Acknowledgments.—The authors wish to express their appreciation of the valuable comments of Dr. Edna M. F. Roe of the Chester Beatty Research Institute, London, England, and of Dr. R. Norman Jones of the National Research Council, Ottawa, Canada, on the problems of spectrophotometric analysis of the stilbene conjugates.

(12) H. J. Creech and H. F. Havas, in preparation.

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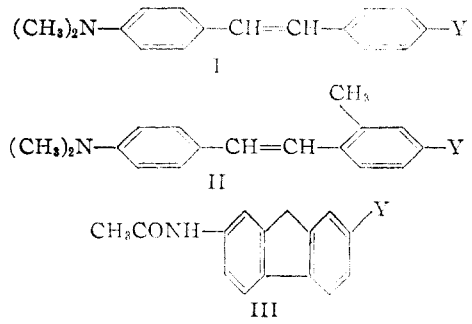
[CONTRIBUTION FROM THE INSTITUTE FOR CANCER RESEARCH AND THE LANKENAU HOSPITAL RESEARCH INSTITUTE]

Isocyanates of Dimethylaminostilbenes and Acetylaminofluorene[1]

BY RICHARD M. PECK AND HUGH J. CREECH

The synthesis of isocyanates of certain systemic carcinogens represents the initial step in an extension of a research program involving immunochemical studies of carcinogen–protein conjugates. 4-Dimethylaminostilbenyl-4'-isocyanate was prepared in 61% yield by the interaction of phosgene with 4-dimethylamino-4'-aminostilbene. 4-Dimethylamino-4'-amino-2'-methylstilbene, which had been prepared in 36% yield by a two-step process from 4-nitro-*o*-xylene and *p*-dimethylaminobenzaldehyde, was converted into the corresponding isocyanate in 79% yield. 2-Acetylaminofluorenyl-7-isocyanate was obtained from the amine in 63% yield. The isocyanates were characterized by conversion into their ethyl urethans.

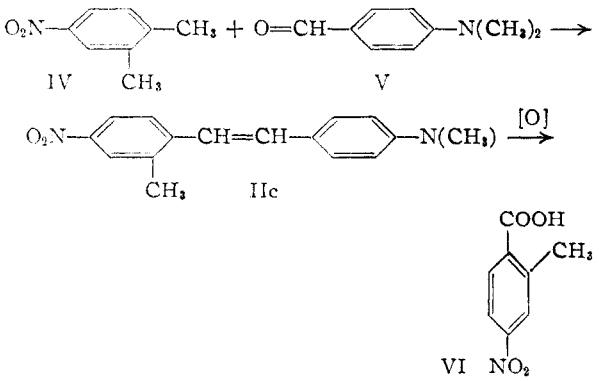
From studies of the potentialities of carcinogen–protein conjugates in the protection of animals against localized carcinogenesis due to polycyclic aromatic hydrocarbons,[2] it was thought desirable to investigate conjugates containing as prosthetic groups the systemic carcinogens 4-dimethylaminostilbene (Ia), 2'-methyl-4-dimethylaminostilbene (IIa) and 2-acetylaminofluorene (IIIa).[3]



(a) Y = H; (b) Y = NCO; (c) Y = NO_2; (d) Y = NH_2

Because of the advantages shown by the carbamido linkage in effecting conjugation of carcinogens with proteins,[4] the synthesis of isocyanates (Ib–IIIb) of these carcinogens was undertaken. In this series, the isocyanate group, obtained in the standard manner by the reaction of the appropriate amine with phosgene,[5] was introduced at a location distant from the biologically important substituted amino group. The conjugation of these isocyanates with amino acids and proteins is described in the accompanying paper[6]; the immunochemical properties of the conjugates are described elsewhere.[7]

The preparation of the compound IIc was carried out in the following manner



That the condensation of *p*-dimethylaminobenzaldehyde (V) had occurred with the methyl group para to the nitro group of 4-nitro-*o*-xylene (IV) to give IIc was demonstrated by oxidation of the

(1) This research was supported in part by a Grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

(2) H. J. Creech, *Acta Unio int. contra cancrum*, **6**, 451 (1949).

(3) A. Haddow, R. J. C. Harris, G. A. R. Kon and E. M. F. Roe, *Phil. Trans. Royal Soc. of London*, **241**, 147 (1948); F. Bielschowsky, *Brit. Med. Bull.*, **4**, 382 (1947).

(4) H. J. Creech and R. N. Jones, THIS JOURNAL, **63**, 1661, 1670 (1941); H. J. Creech, E. L. Oginsky and F. S. Cheever, *Cancer Research*, **7**, 290 (1947).

(5) H. J. Creech, THIS JOURNAL, **63**, 576 (1941).

(6) H. J. Creech and R. M. Peck, *ibid.*, **74**, 463 (1952).

(7) H. J. Creech and H. F. Havas, in preparation.

product to the known compound 4-nitro-*o*-toluic acid (VI).

Experimental

4-Dimethylamino-4'-aminostilbene[8] **(Id).**—To a solution of 89.5 g. (0.397 mole) of stannous chloride dihydrate in 262 ml. of concentrated hydrochloric acid was added 32 g. (0.12 mole) of 4-dimethylamino-4'-nitrostilbene.[8] The mixture was swirled and heated to the boiling point for ten minutes after solution had been effected. When the mixture had been allowed to cool to the point of turbidity, it was poured into a cold, stirred solution of 360 g. of potassium hydroxide pellets in 600 ml. of water. Since the product did not separate cleanly, 300 ml. of benzene was added. The mixture was stirred and heated until there were only two liquid phases and then was cooled in an ice-bath. After addition of 600 ml. of Skellysolve "B," the precipitate was removed by filtration using a sintered glass funnel and washed with distilled water and with petroleum ether. The dried product was recrystallized from benzene; the yield was 21.9 g. (77%), m.p. 172–173°. A sample was submitted for analysis. *Anal.* Calcd. for $C_{16}H_{18}N_2$: C, 80.6; H, 7.6; N, 11.7. Found: C, 80.6, 80.8; H, 7.7, 7.6; N, 12.0, 11.8.

4-Dimethylamino-2'-methyl-4'-nitrostilbene (IIc).—A mixture of 46.6 g. (0.309 mole) of 4-nitro-*o*-xylene,[9] 10 ml. of piperidine and 46.8 g. (0.314 mole) of *p*-dimethylaminobenzaldehyde was heated overnight under reflux in an oil-bath maintained at 150°. The volatile material (mainly piperidine and water formed in the reaction) was removed by stirring and application of vacuum. Five ml. of piperidine was replaced and the mixture was heated for an additional five hours. After removal of volatile material *in vacuo*, 100 ml. of benzene and 20 ml. of 30–60° petroleum ether were added. After cooling and filtering, 9.6 g. of product was obtained. The solvent was removed from the mother liquor, 10 ml. of piperidine was added, and the mixture was again refluxed for 24 hours in a 150° oil-bath. On removal of volatile material *in vacuo* and addition of benzene and petroleum ether with cooling, a second crop of 18 g. of material was obtained. Reworking of the mother liquor in the same way gave 12.8 g. and 10.1 g. as third and fourth crops. The total amount (50.5 g.) was recrystallized from benzene–petroleum ether to give 40.6 g. (over-all yield 46.5%) of product, m.p. 174.8–175.8°, s. 173°. Further working of the filtrate gave 7.5 g. of cruder material. A sample purified for analysis melted at 176.0–177.0°. *Anal.* Calcd. for $C_{17}H_{18}N_2O_2$: C, 72.3; H, 6.4; N, 9.9. Found: C, 72.8, 72.9; H, 6.8, 6.9; N, 9.4, 9.5. Alkaline permanganate oxidation of a sample of this material gave a nitrotoluic acid melting at 151.5–152.0°, identified as 4-nitro-*o*-toluic acid (VI); elimination of water, therefore, occurred at the methyl group para to the nitro group of IV.

4-Dimethylamino-4'-amino-2'-methylstilbene (IId).—To a solution of 90 g. (0.40 mole) of stannous chloride dihydrate in 120 ml. of concentrated hydrochloric acid and 35 ml. of water was added 28.2 g. (0.10 mole) of 4-dimethylamino-2'-methyl-4'-nitrostilbene. After three hours stirring at room temperature, the mixture was heated to the boiling point with stirring. After 30 minutes at the boiling point, the solution was clear; boiling was continued for five minutes and the mixture was cooled overnight. The supernatant was discarded, and the solid cake of tin double salt was stirred with hot 10% sodium hydroxide solution and benzene. The process of decomposition was rather slow; when the greater part of the solid had disappeared, the liquid phases were siphoned and separated,[10] and the water layer was extracted with benzene. The remaining solid was treated twice more with 10% sodium hydroxide solution and benzene, after which all of the complex had been decomposed. The combined benzene layers and extracts were concentrated to 150 ml., decolorized with Norit and filtered while hot. Gradual addition of an equal volume of 30–60° petroleum ether followed by cooling overnight gave 21.9 g. (87% yield) of crystalline product. This was dissolved in excess methanol, treated with Norit, filtered, concentrated to about 300 ml., and cooled thoroughly after addition of about 100 ml. of distilled water. Filtration gave 19.8 g. of product (78.5% yield) which melted at 129.5–131.0°, s. 126.5°. An analytical sample was recrystallized to a constant melting point of 129.8–131.0°. *Anal.* Calcd. for $C_{17}H_{20}N_2$: C, 81.0; H, 8.0; N, 11.1. Found: C, 80.4, 80.7; H, 8.3, 8.2; N, 10.7, 10.9.

4-Dimethylaminostilbenyl-4'-isocyanate (Ib).—Two hundred and fifteen milliliters of toluene (dried over calcium hydride) was cooled in ice-water while phosgene gas was bubbled through. An increase in volume of about 25 ml. showed an increase in weight of 34 g. (0.34 mole) of phosgene. This solution was stirred vigorously while a solution of 5 g. (0.0198 mole) of 4-dimethylamino-4'-aminostilbene in 250 ml. of dry benzene was added rapidly. The thoroughly mixed suspension was concentrated rapidly to about 180 ml. and cooled. About 20 ml. of Skellysolve "B" was added to the mixture. Filtration gave 4.2 g. of crude product, m.p. 206–208°. Recrystallization from benzene, with the use of freshly ignited Norit, gave 3.4 g. (61% yield) of recrystallized product, m.p. 206.5–207.8°. A sample of m.p. 204.6–205.5° previously obtained was analyzed. *Anal.* Calcd. for $C_{17}H_{16}N_2O$: C, 77.3; H, 6.1; N, 10.6. Found: C, 78.1, 77.8; H, 6.0, 5.9; N, 10.4, 10.2.

4-Dimethylamino-2'-methylstilbenyl-4'-isocyanate (IIb).—The reaction of 5 g. of 4-dimethylamino-4'-amino-2'-methylstilbene with 34 g. of phosgene was carried out as described above except that the reaction mixture was concentrated to 30 ml. and four volumes of Skellysolve "B" were added. The mother liquor was decanted from the crystals formed during overnight cooling and the product was recrystallized from a dry mixture of 10% benzene–90% Skellysolve B, after clarification with freshly ignited Norit. In the case of this compound, two crystalline forms, one feathery and one compact, were formed. The former melted at 113.5–114.9°; the latter melted at 112–114.2°; the mixed melting point was 113–114.9°, s. 111.5°. The absorption spectra of the two products were identical. The light crystals weighed 2.85 g.; the other modification weighed 1.50 g.; the total yield was 79%. A sample of melting point 114–114.9°, purified by sublimation, was analyzed. *Anal.* Calcd. for $C_{18}H_{18}N_2O$: C, 77.7; H, 6.5; N, 10.1. Found: C, 78.0, 77.9; H, 6.3, 6.3; N, 10.1, 9.9.

2-Acetylaminofluorenyl-7-isocyanate (IIIb).—To a stirred solution of about 6.8 g. (0.068 mole) of phosgene in 50 ml. of dry toluene was added rapidly a solution of 1.0 g. of freshly sublimed 7-amino-2-acetylaminofluorene[11] in 80 ml. of warm, purified dioxane. The mixture was heated to boiling and refluxed for ten minutes, by which time the precipitate had disappeared. The solution was concentrated *in vacuo* to about 15 ml. and an equal volume of Skellysolve B was added. The filtered product weighed about 0.74 g. Recrystallization from dioxane–Skellysolve, with clarification with freshly ignited Darco, gave 0.70 g. (63% yield) of product with m.p. about 220° (turbid melt). Recrystallization from dioxane–Skellysolve and from toluene gave a pure sample, m.p. 216–220° (*in vacuo*, the compound melted at about 205–210° with gas evolution). *Anal.* Calcd. for $C_{16}H_{12}N_2O_2$: C, 72.7; H, 4.6; N, 10.6. Found: C, 73.2, 73.2; H, 4.8, 4.7; N, 9.9, 10.0.

Ethyl 4-Dimethylaminostilbenyl-4'-carbamate.—A solution of 100 mg. of 4-dimethylamino-4-stilbenyl-4'-isocyanate in 10 ml. of absolute ethanol was refluxed overnight. A small amount of insoluble material was removed by filtration and the product was recovered by concentration and dilution with Skellysolve "B." The yield of urethan was 70 mg. (58%), m.p. 190–191.5° dec. Recrystallization to constant melting point gave an analytical sample melting at 190.5–192° dec. (lower with slow heating). *Anal.* Calcd. for $C_{19}H_{22}N_2O_2$: C, 73.6; H, 7.2; N, 9.0. Found: C, 73.4, 73.2; H, 7.3, 7.1; N, 9.0, 8.9.

Ethyl 4-Dimethylamino-2'-methylstilbenyl-4'-carbamate.—This preparation was made similarly in a 68% yield from 100 mg. of 4-dimethylamino-2'-methylstilbenyl-4'-isocyanate. The analytical sample softened at 102° and melted at 102.4–103°. *Anal.* Calcd. for $C_{20}H_{24}N_2O_2$: N, 8.6. Found: N, 8.8, 8.7.

(8) Prepared in 40% yield by a modification of the method of Chardonnens and Heinrick, *Helv. Chim. Acta*, **22**, 1471 (1939).

(9) Prepared in 27% yield according to the method of Karrer, *et al.*, *ibid.*, **18**, 1435 (1935).

(10) The water layer contained a fine yellow precipitate which was not extracted.

(11) M.p. 200–202°. The reduction of 2-acetylamino-7-nitrofluorene was carried out according to the method of Cislak and Hamilton, THIS JOURNAL, **53**, 746 (1931), using, however, zinc activated with a small amount of cupric sulfate and a reduction period of one hour.

Ethyl 2-Acetylaminofluorenyl-7-carbamate.—A solution of 0.15 g. of 2-acetylaminofluorenyl-7-isocyanate in 15 ml. of ethanol was refluxed 10 minutes, diluted, and cooled. On filtration there was obtained 0.10 g. (57% yield) of product. Recrystallization from ethanol gave an analytical sample of m.p. 248.5–250.5°. *Anal.* Calcd. for $C_{18}H_{18}N_2O_3$: N, 9.0. Found: N, 9.2, 9.0.

PHILADELPHIA, PENNA. RECEIVED JULY 25, 1951

[CONTRIBUTION NO. 830 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

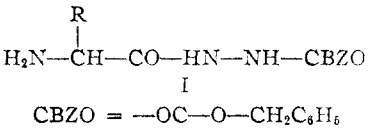
Studies on Polypeptides. III.[1] Novel Routes to α-Amino Acid and Polypeptide Hydrazides

BY KLAUS HOFMANN,[2] ADOLF LINDENMANN,[3] MARGARET Z. MAGEE AND NOORUL HAQ KHAN

A novel procedure for the synthesis of acylated α-amino acid and polypeptide hydrazides is presented. The method involves the synthesis of α-amino acid carbobenzoxyhydrazides, their incorporation into polypeptide derivatives, followed by liberation of the hydrazide group by hydrogenolysis. Three procedures for the preparation of α-amino acid carbobenzoxyhydrazides are described. The transformation of three L-amino acid amides into the respective 4-substituted-2-thio-5-thiazolidones is shown to involve racemization. A method allowing the preparation of phthalyl amino acid and peptide hydrazides is given. A number of acylated tripeptide carbobenzoxyhydrazides were synthesized in good yields by the interaction of acylated dipeptide azides with glycine carbobenzoxyhydrazide. Triglycine hydrazide dihydrochloride was prepared in good yield by the hydrogenation of carbobenzoxytriglycine carbobenzoxyhydrazide.

The conventional method for the preparation of acylated polypeptide hydrazides involves the treatment of acylated peptide esters with hydrazine. Although highly successful with acylated amino acid and dipeptide esters, this method is not generally applicable when the preparation of more highly complex acylated polypeptide hydrazides is desired.

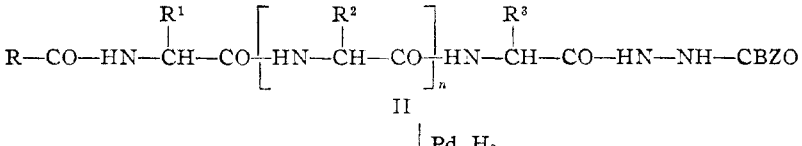
The application to the synthesis of peptide hydrazides of α-amino acid carbobenzoxyhydrazides of the general structure (I) offers distinct advantages.



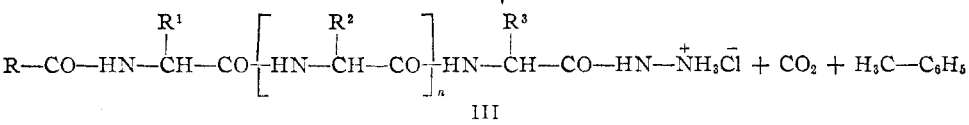
I

CBZO = —OC—O—$CH_2C_6H_5$

These compounds by way of their free amino group may be readily combined with other acylated peptide structures to form polypeptide carbobenzoxyhydrazides of the general formula (II)



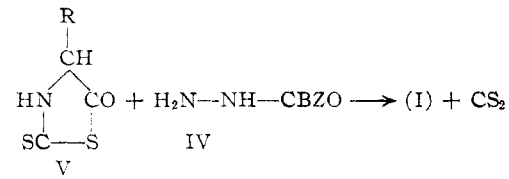
II



III

Hydrogenolysis uncovers the hydrazide function thus producing an acylated polypeptide hydrazide (III), which may be attached to other peptide derivatives through its azide. This novel method for the synthesis of hydrazides allows the introduction of a potential hydrazide group into a peptide moiety at the monoamino acid stage, thus avoiding the exposure of complex sensitive peptides to hydrazine. The present communication describes procedures for the preparation of α-amino acid carbobenzoxyhydrazides and illustrates some uses of these compounds. The key reagent for this purpose was carbobenzoxyhydrazine (IV)[4] which has not been used hitherto in the preparation of hydrazides.

The reaction of 2-thio-5-thiazolidones (V) with amines or amino acid esters leads, under suitable conditions, to the formation of amino acid amides or dipeptide esters.[5] This scheme seemed applicable to the synthesis of α-amino acid carbobenzoxyhydrazides. Heating of an equimolar mixture of 2-thio-5-thiazolidone[6] (V, R = H) and carbobenzoxyhydrazine in glacial acetic acid led to the evolution of carbon disulfide. From the reaction mixture it was possible to isolate glycine carbobenzoxyhydrazide (I, R = H) in the form of its well-crystallized hydrochloride. The elementary analysis and the presence of a free primary amino group were in accord with the expected structure.



IV

V

The amides of DL-alanine and of DL-phenylalanine were then converted into the corresponding 2-thio-

(1) For paper No. II see K. Hofmann, M. Z. Magee and A. Lindenmann, THIS JOURNAL, **72**, 2814 (1950).

(2) This investigation was supported by grants from the U. S. Public Health Service, The Rockefeller Foundation in New York and Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

(3) Postdoctorate Research Fellow from the University of Basel, Switzerland.

(4) N. Rabjohn, THIS JOURNAL, **70**, 1181 (1948).

(5) I. Heilbron, *J. Chem. Soc.*, 2099 (1949).

(6) A. H. Cook, I. Heilbron and A. L. Levy, *ibid.*, 201 (1948).