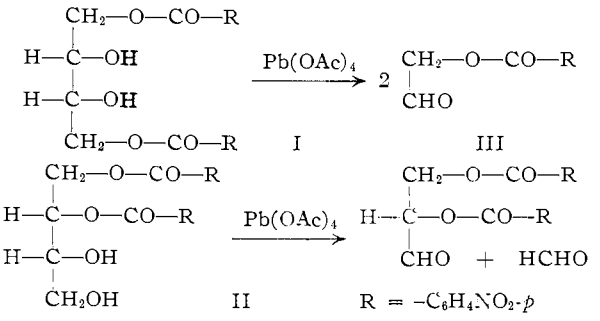

benzoylglycolic aldehyde (III) thus produced was characterized by the formation of its crystalline 2,4-dinitrophenylhydrazone.

Experimental

Preparation of 1,4-Bis-*O*-*p*-nitrobenzoylerythritol.[9]—To a solution of erythritol (1.525 g.) in pyridine (30 ml.), well cooled in an ice-bath, a solution of *p*-nitrobenzoyl chloride (4.635 g.) in pyridine (30 ml.) was added slowly with vigorous stirring. After the addition had been completed, the reaction mixture was allowed to come slowly to room temperature by standing in the melting ice-bath overnight. After removing most of the pyridine by distillation *in vacuo*, the residual thin sirup was poured, with stirring, into ice-water (1000 ml.) containing sodium bicarbonate (4.0 g.). The precipitated ester was filtered, washed free of pyridine with water and dried on a porous plate. The dried product (1.49 g.) was dissolved in boiling 95% ethanol (300 ml.) and the resulting solution was filtered hot in order to separate a small amount of insoluble residue. The clear filtrate was allowed to evaporate in an open beaker for 4 days at room temperature. The compact clusters of small needles thus formed (1.05 g.) gave upon recrystallization from absolute ethanol (250 ml.) 1,4-bis-*O*-*p*-nitrobenzoylerythritol, m.p. 202–203°.

Anal. Calcd. for $C_{18}H_{16}O_{10}N_2$: C, 51.4; H, 3.8; N, 6.7. Found: C, 51.5; H, 3.9; N, 6.8.

Oxidation with Lead Tetraacetate.—To a solution of 1,4-bis-*O*-*p*-nitrobenzoylerythritol (84 mg., 0.0002 mole) in glacial acetic acid (25 ml.) was added 0.1033 *N* lead tetraacetate in glacial acetic acid (15 ml.). The mixture was adjusted to a volume of 50 ml. with glacial acetic acid and then heated on the water-bath at 85° for 1.5 hours. At the end of this time an aliquot of the reaction mixture was added to an aqueous sodium acetate–potassium iodide buffer solution and the liberated iodine was titrated with a standard sodium thiosulfate solution.[10] This titration showed that 0.97 mole of lead tetraacetate had been consumed per mole of 1,4-bis-*O*-*p*-nitrobenzoylerythritol.

Test for the Presence of Formaldehyde in the Oxidation Product.—An aliquot (30 ml.) of the reaction mixture from the previous experiment was treated with 60 ml. of aqueous sodium acetate–potassium iodide buffer solution[10] and the liberated iodine was reduced with sodium thiosulfate solution. The resulting solution was distilled *in vacuo* on the water-bath (40–50°). The first few ml. of aqueous distillate gave a negative Schiff test.

For the purpose of comparison, a 30-ml. aliquot of a solution of formaldehyde (0.0002 mole, corresponding to 0.0002 mole of 1,4-bis-*O*-*p*-nitrobenzoylerythritol) in 50 ml. of glacial acetic acid which had been heated on the water-bath at 85° for 1.5 hours, was treated with 60 ml. of the aqueous sodium acetate–potassium iodide buffer solution[10] and distilled *in vacuo* (bath, 40–50°). The first few ml. of distillate gave a positive Schiff test.

Preparation of *O*-*p*-Nitrobenzoylglycolic Aldehyde 2,4-Dinitrophenylhydrazone.—To a suspension of 1,4-bis-*O*-*p*-nitrobenzoylerythritol (0.5 g.) in benzene (30 ml.) was added one molecular equivalent of lead tetraacetate (0.4294 g.). The mixture was refluxed for 1.5 hours during which time the crystals of the ester disappeared and white lead acetate separated. The cool solution was filtered to remove the lead acetate and shaken with saturated sodium bicarbonate solution to remove acetic acid. The benzene solution was dried over anhydrous calcium chloride and evaporated *in vacuo* to give a white crystalline residue (0.3758 g.) which gave a positive Schiff test. The crystalline material, which was not purified further since it decomposed slowly on standing, was insoluble in water but suspended particles of it turned a deep violet with Schiff reagent. It did not reduce boiling Fehling solution.

To a solution of the crystalline material (0.3008 g.) in 95% ethanol (30 ml.) was added glacial acetic acid (3 ml.) followed by 2,4-dinitrophenylhydrazine (0.3 g.). The reaction mixture was agitated, occasionally, over a period of about 30 min. during which time the red crystals of 2,4-dinitrophenylhydrazine dissolved and bright yellow crystals separated. The reaction was completed by refluxing for 15–20 min. After allowing the reaction mixture to cool to room temperature, the 2,4-dinitrophenylhydrazone of glycolic aldehyde *O*-*p*-nitrobenzoate was filtered off and washed with 95% ethanol (yield 0.4487 g.). It was recrystallized from boiling 86% (v./v.) aqueous acetic acid (35 ml.). Upon standing, large orange needles separated followed by canary-yellow fluffy crystal masses. By suspending the combined crystals in ether it was possible to decant, almost quantitatively, the suspended yellow crystals from the heavier orange crystals. The orange crystals and the yellow crystals had the same melting point, 189.5–190.5°, and a mixed melting point gave no depression. When the orange crystals were recrystallized from chloroform–petroleum ether, only yellow crystals were formed.

Anal. Calcd. for $C_{12}H_{11}O_8N_5$: C, 46.3; H, 2.9; N, 18.00. Found (for orange crystals): C, 46.3; H, 3.3; N, 17.9. Found (for yellow crystals): C, 46.7; H, 3.3; N, 18.1.

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(9) *Cf.* A. Einhorn and F. Hollandt, *Ann.*, **301**, 95 (1898).

(10) R. C. Hockett and W. S. McClenahan, THIS JOURNAL, **61**, 1670 (1939).

Reactions of Vanillin and its Derived Compounds. XXV.[1] Hydrazides of Vanillic and Related Acids[2]

BY IRWIN A. PEARL AND DONALD L. BEYER

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During the course of our studies on lignin model compounds and on vanillin derivatives we had occasion to prepare many carboxylic acids related to vanillic acid. Recent developments in the pharmaceutical field indicated that hydrazides of these acids might have activity against certain microorganisms. Accordingly, the methyl or ethyl ester of acids previously reported in our vanillin and lignin studies was treated in ethanol with an excess of hydrazine hydrate. In most cases the desired hydrazide was obtained. Data for these hydrazides are found in Table I.

In some reactions anomalous products were formed. Reaction of ethyl orthovanillate with hydrazine hydrate in the regular manner yielded the hydrazine salt of orthovanillic acid hydrazide. If the solution was acidified with sulfuric acid after reaction, the hemisulfate hydrate of orthovanillic acid hydrazide was obtained. The hydrazine salt of orthovanillic acid hydrazide also was obtained if the methyl or ethyl ester of orthovanillic acid allyl ether was treated in the same manner with hydrazine hydrate. The cleavage of the allyl group in these compounds by the hydrazinolysis reaction and lack of cleavage of the allyl group in the case of

(1) For paper XXIV of this series, see THIS JOURNAL, **77**, 757 (1955); XXVI, *ibid.*, **77**, 2826 (1955).

(2) The results reported here are from a research program at this Institute, sponsored by the Sulphite Pulp Manufacturers' Research League. Acknowledgment is made for their permission to publish these results.

TABLE I

HYDRAZIDES OF CARBOXYLIC ACIDS

Acid	Yield, %	M.p., [a] °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
5-Allyl-2-hydroxy-3-methoxybenzoic	84	128–129	$C_{11}H_{14}N_2O_3$	59.45	59.45	6.35	6.40
5-Allyl-4-hydroxy-3-methoxybenzoic	91	196–197	$C_{11}H_{14}N_2O_3$	59.45	59.48	6.35	6.44
4-Allyloxy-3-methoxybenzoic	95	114–115	$C_{11}H_{14}N_2O_3$	59.45	59.63	6.35	6.38
4-Benzyloxy-3-methoxybenzoic	93	152–153	$C_{15}H_{16}N_2O_3$	66.16	66.08	5.92	5.91
3,4-Dihydroxybenzoic	72	268[b]	$C_7H_8N_2O_3$	50.00	49.92	4.80	4.86
2,3-Dimethoxybenzoic	94	81–82	$C_9H_{12}N_2O_3$	55.09	54.96	6.17	6.17
3,4-Dimethoxybenzoic[c]	88	137–138	$C_9H_{12}N_2O_3$	55.09	55.02	6.17	6.14
3,5-Dimethoxy-4-hydroxybenzoic	100	156–157	$C_9H_{12}N_2O_4$	50.94	51.04	5.70	5.64
3-Ethoxy-4-hydroxybenzoic	95	192–193	$C_9H_{12}N_2O_3$	55.09	55.40	6.17	6.31
4-Hydroxy-3-methoxyphenylacetic	90	158–159	$C_9H_{12}N_2O_3$	55.09	55.20	6.17	6.26
3,4,5-Trimethoxybenzoic	100	157–158[d]	$C_{10}H_{15}N_2O_{4.5}$	51.06	51.25	6.43	6.53

[a] From ethanol. [b] With gas evolution. [c] No solvent was employed in this reaction. [d] Hemihydrate. The anhydrous compound, m.p. 159°, was prepared by R. O. Pepe [*J. prakt. Chem.*, 126, 241 (1930)].

the ethyl ester of vanillic acid allyl ether cannot be explained. Reaction of esters of 5-chloro or 5-bromovanillic acid yielded only hydrazine salts of the original esters. In the reaction with ethyl 5-nitroörthoveratrate, hydrazine hydrate again acted as a dealkylating agent and yielded an hydroxy-methoxybenzoic acid, the position of demethylation of which was not proved, but which is probably 3-hydroxy-2-methoxybenzoic acid.

Reactions of the ethyl esters of ferulic, 5-chloro-ferulic and 5-propenylvanillic acids with hydrazine hydrate in ethanol gave only oily mixtures from which no crystalline materials could be isolated.

All reaction products were tested *in vitro* against three important representative pathogenic organisms, *Staphylococcus aureus*, *Klebsiella pneumoniae* and the BCG strain of *Mycobacterium tuberculosis*. Data are given in Table II. It is obvious from these data that the hydrazides of this study do not possess great activity against these representative microörganisms.

TABLE II

ANTIBACTERIAL DATA ON HYDRAZIDES AND RELATED COMPOUNDS

Compound	Minimum inhibiting concn., mg./ml. *Staph. aureus*	*Klebs. pneum.*	BCG strain *M. tubercl.*
5-Allyl-2-hydroxy-3-methoxybenzhydrazide	0.03+[a]	0.03+	0.03
5-Allyl-4-hydroxy-3-methoxybenzhydrazide	.10+	.10+	.10+
4-Allyloxy-3-methoxybenzhydrazide	.03+	.03+	.03+
4-Benzyloxy-3-methoxybenzhydrazide	.03+	.03+	.025
3,4-Dihydroxybenzhydrazide	.15+	.15+	.08
2,3-Dimethoxybenzhydrazide	.15+	.15+	.15+
3,4-Dimethoxybenzhydrazide	.15+	.15+	.15
3,5-Dimethoxy-4-hydroxybenzhydrazide	.08	.15+	.06
3-Ethoxy-4-hydroxyhenzhydrazide	.10+	.10+	.10+
4-Hydroxy-3-methoxyphenylacetic acid hydrazide	.15+	.15+	.15+
3,4,5-Trimethoxybenzhydrazide	.03+	.03+	.03+
2-Hydroxy-3-methoxybenzhydrazide, hemisulfate hydrate	.15+	.15	.012
2-Hydroxy-3-methoxybenzhydrazide, hydrazine salt	.12	.08	.006
Ethyl 5-bromovanillate	.15+	.15+	.10
3-Hydroxy-2-methoxy-5-nitrobenzoic acid	.03+	.03+	.03+
Isobutyl 5-chlorovanillate, hydrazine salt	.10+	.03+	.03+

[a] + indicates activity without complete inhibition.

Experimental

All melting points are uncorrected.

General Procedure for Hydrazides.—A mixture of one mole of the ethyl ester of the desired acid, 2.5 moles of 85% hydrazine hydrate and 300 cc. of 95% ethanol was heated to boiling under reflux for six hours and allowed to stand overnight. The reaction mixture was concentrated from a steam-bath under reduced pressure until nothing further distilled, and the residue was poured into two liters of cold water with stirring. The precipitate was filtered, washed with water, dried and recrystallized from ethanol.

Hydrazinolysis of Ethyl 2-Hydroxy-3-methoxybenzoate.—The residue after concentration as in the general procedure above gave a clear solution when poured into water. The solution was acidified with sulfuric acid and concentrated to one-half volume. The white precipitate was filtered and recrystallized from ethanol to yield white crystals of the hemisulfate hydrate of 2-hydroxy-3-methoxybenzhydrazide, melting at 200–201°.

Anal. Calcd. for $C_8H_{13}N_2O_6S_{0.5}$: C, 38.55; H, 5.26; N, 11.24; S, 6.4; CH_3O, 12.4. Found: C, 38.45; H, 5.31; N, 11.09; S, 6.6; CH_3O, 12.1.

In another similar experiment the reaction mixture was cooled after six hours of boiling. The precipitate was filtered, washed with ethanol, and recrystallized from ethanol to give granular crystals of the hydrazine salt of 2-hydroxy-3-methoxybenzhydrazide melting at 141–142°.

Anal. Calcd. for $C_8H_{14}N_4O_3$: C, 44.85; H, 6.59. Found: C, 45.00; H, 6.65.

Hydrazinolysis of Ethyl 2-Allyloxy-3-methoxybenzoate.—The reaction mixture was concentrated to remove ethanol and cooled. The precipitate was filtered, washed with petroleum ether, and recrystallized from ethanol to yield granular crystals of the hydrazine salt of 2-hydroxy-3-methoxybenzhydrazide melting at 141–142° and not depressing a mixed melting point with the product obtained from ethyl 2-hydroxy-3-methoxybenzoate. The same product was obtained from the hydrazinolysis of methyl 2-allyloxy-3-methoxybenzoate.

Anal. Calcd. for $C_8H_{14}N_4O_3$: C, 44.85; H, 6.59. Found: C, 44.92; H, 6.58.

Hydrazinolysis of Ethyl 5-Bromovanillate.—A precipitate separated in the reaction mixture on cooling. The entire mixture was stirred into excess water, and the precipitate was filtered, washed with water, air-dried, and recrystallized from benzene to yield white crystals melting at 151–152° with gas evolution. Recrystallization from water gave ethyl 5-bromovanillate melting at 123–124°. The 151–152° melting compound appears to be the hydrazine salt of ethyl 5-bromovanillate less one molecule of water. The yield was 55%.

Anal. Calcd. for $C_{10}H_{13}BrN_2O_3$: C, 41.54; H, 4.53. Found: C, 41.37; H, 4.56.

Hydrazinolysis of Isobutyl 5-Chlorovanillate.—The residue after concentration of the reaction mixture was stirred into water. The precipitate was filtered, washed with water, and air-dried to give a crude product which was recrystallized twice from benzene to yield tiny white crystals of the hydrazine salt of isobutyl 5-chlorovanillate melting at 129–130°.

Anal. Calcd. for $C_{12}H_{19}ClN_2O_4$: C, 49.57; H, 6.59. Found: C, 49.67; H, 6.67.

Hydrazinolysis of Ethyl 2,3-Dimethoxy-5-nitrobenzoate.—The red colored reaction mixture was concentrated under

reduced pressure and stirred into excess water. The still clear solution was acidified with dilute hydrochloric acid, and the resulting yellow precipitate was filtered, washed with water, and recrystallized to yield fluffy yellow crystals melting at 296–297°. The product appears to be a nitro-hydroxy-methoxy-benzoic acid.

Anal. Calcd. for $C_8H_7NO_6$: C, 45.08; H, 3.31. Found: C, 45.10; H, 3.32.

Inasmuch as Bolliger and Reuter[3] reported a melting point of 220° for 2-hydroxy-3-methoxy-5-nitrobenzoic acid, this compound is probably 3-hydroxy-2-methoxy-5-nitrobenzoic acid.

Acknowledgment.—The authors wish to thank Mr. Harold Willemsen for the analyses and Dr. R. Donovick of the Squibb Institute for Medical Research for the values of the inhibiting concentrations for the pathogenic microörganisms reported in this paper.

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Azo Derivatives of Some Aromatic Poly-α-amino Acids

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In this paper the synthesis and properties of some azo derivatives of poly-α-amino acids containing aromatic amino acid residues are described. These compounds may serve as models in the study of azoproteins, which are widely used in immunological studies as well as in the investigation of the chemical and biological properties of proteins.[1,2] The colored azopolypeptides obtained represent a new group of polymeric dyes.[3] Their synthesis permits the attachment through an azo link of different compounds to polyamino acids containing residues of tyrosine, histidine, tryptophan or *p*-aminophenylalanine.

Poly-3-(*p*-nitrophenylazo)-L-tyrosine and poly-3,5-di-(*p*-bromophenylazo)-L-tyrosine were prepared by coupling poly-L-tyrosine[4] with *p*-nitrobenzene or *p*-bromobenzene diazonium salts, respectively. For comparison 3,3′-di-(*p*-nitrophenylazo)-DL-tyrosine anhydride was prepared. Poly-*p*-(1-hydroxynaphthyl-4-azo)-DL-phenylalanine was obtained by coupling α-naphthol with diazotized poly-*p*-amino-DL-phenylalanine.[5] The diazonium salt derived from poly-*p*-aminophenylalanine also was coupled with tyrosine and with polytyrosine.

The absorption spectrum of poly-3-(*p*-nitrophenylazo)-tyrosine (*n* average 30) was determined at *p*H 13 between 2500 and 6000 Å. Two characteristic peaks were found, at 3460 Å. (residue molar extinction coefficient, ε 10000) and at 5300 Å. (ε 6350). An identical spectrum was found for 3,3′-di-(*p*-nitrophenylazo)-tyrosine anhydride. Two peaks, at 3460 Å. (ε 7250) and at 5300 Å. (ε 6350) were found in the absorption spectrum of 3-(*p*-nitrophenylazo)-*p*-cresol,[6] determined for comparison. The absorption spectrum of poly-*p*-(1-hydroxynaphthyl-4-azo)-phenylalanine (*n* average 20) in ethanolamine shows, between 2500 and 6000 Å., one characteristic peak, at 5180 Å. (ε 10000).

A copolymer of L-tyrosine and L-lysine, in a molar ratio of 1:10, was coupled with *p*-nitrobenzene diazonium chloride. The azopolypeptide obtained resembled polylysine in its solubility, influence on blood clotting[7] and antibacterial activity.[8] A copolymer of *p*-aminophenylalanine and L-aspartic acid, in a molar ratio of 1:9, was diazotized and coupled with α-naphthol. The colored derivative thus obtained resembled polyaspartic acid in solubility and biological activity.[7,8] These findings indicate that azo derivatives of α-amino acid copolymers containing a small percentage of aromatic amino acids might be used as tagged compounds in certain biological investigations.

Experimental

All melting points are uncorrected. The coupling reactions were carried out in 1*N* sodium hydroxide or sodium carbonate at 0–5° in the usual way. Absorption measurements were made on a Beckman model DU spectrophotometer, at approximately 25°.

3,3′-Di-(*p*-nitrophenylazo)-DL-tyrosine anhydride was prepared by coupling DL-tyrosine anhydride[9] (1 mole) with *p*-nitrobenzene diazonium chloride (2 moles), precipitated from reaction mixture with dilute hydrochloric acid and recrystallized from *t*-butyl alcohol; m.p. 243–245°, yield 91%.

Anal. Calcd. for $C_{30}H_{24}N_8O_5$: C, 57.7; H, 3.9; N, 17.9. Found: C, 57.3; H, 3.9; N, 17.6.

The brown azo compound dissolves readily in acetone, pyridine, butylamine, ethanolamine and ethylenediamine, is sparingly soluble in ethanol, chloroform and dioxane, and is insoluble in water, ether, benzene and carbon tetrachloride. Its solutions in aqueous or alcoholic sodium hydroxide are red-violet.

The azo derivative of tyrosine anhydride moved as a single red-violet band on an activated alumina column, when 1 *N* sodium hydroxide was passed through the column. When the substance was reduced and hydrolyzed for 12 hours in a boiling solution of stannous chloride (10%) in 6 *N* hydrochloric acid, and an aqueous solution of the dried reaction mixture was run on a paper chromatogram developed with *n*-butyl alcohol–water–acetic acid (4:5:1), only one spot, with R_f 0.28, was obtained upon spraying with ninhydrin. One spot, with R_f 0.80, was obtained when the paper chromatogram was developed with *n*-propyl alcohol–0.6% aqueous ammonia (2:1). Paper electrophoresis of the above hydrolysate was carried out on Whatman No. 1 filter paper in acetate buffer of *p*H 3.6 and ionic strength 0.2, at a potential gradient of 10 *V*/cm. at room temperature. A single spot, at a distance of 8.7 cm. from the origin toward the cathode, was revealed, after two hours, with ninhydrin. Tyrosine did not move from the origin under the same conditions. The homogeneity of the azo derivative of the tyrosine anhydride, the lack of tyrosine in the hydrolysate of the reduction product, as well as the appearance of one spot, most probably corresponding to that of 3-aminotyrosine, in the chromatographic and electrophoretic experiments, support the symmetric formula of 3,3′-di-(*p*-nitrophenylazo)-DL-tyrosine anhydride suggested.

Poly-3-(*p*-nitrophenylazo)-L-tyrosine was prepared by coupling poly-L-tyrosine[4] (*n* average 30) with *p*-nitrobenzene-diazonium chloride (one mole for each mole of tyrosine residue); precipitated with dilute hydrochloric acid, purified by dissolving in aqueous ammonia and reprecipitating with acetic acid; yield 93%.

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(2) R. Herriott, *Advances in Protein Chem.*, **3**, 169 (1947).

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