Methyl 3α-Acetoxy-11-ketoetiocholanate (LXXXIV, R = Ac, R′ = OMe). — Methyl 3,11-diketoetiocholanate (LXXXIII, R = H)[110] (40 mg.) dissolved in ethanol (6 cc.) at 0° was reduced with sodium borohydride (40 mg.) in ethanol (1 cc.) at 0° for 20 hours.[111] Isolation with ether in the usual way gave a product which was dissolved in 90% methanol (5 cc.), digitonin (100 mg.) in 90% methanol (5 cc.) was added, and the cloudy solution was left for one hour. The solvent was evaporated at the water pump, and the residue was well extracted with ether. Evaporation of the solvent gave a product (35 mg.) which when seeded with methyl 3α-hydroxy-11-ketoetiocholanate[61] only partially solidified. It was dissolved in pyridine (1 cc.) and acetylated with acetic anhydride (0.5 cc.) in the usual way. The product was dissolved in benzene–petroleum ether (1:1) and poured onto a short alumina column (*ca.* 1 g.), which was washed with the same solvent mixture (50 cc.) and then with methanol (30 cc.). The non-polar solvents gave a solid material (26 mg.) which on crystallization from ether–petroleum ether yielded methyl 3α-acetoxy-11-ketoetiocholanate (LXXXIV, R = Ac, R′ = OMe) (18 mg.) as laths, m.p. 150–154° (Kof.). The methanol fraction yielded a crystalline material (12 mg.), m.p. 176–181° (Kof.), probably methyl 3α-acetoxy-11β-hydroxyetiocholanate[112] formed by reduction of both the keto groups of (LXXXIII, R = H). It was combined with the mother liquors from the crystallization of (LXXXIV, R = Ac, R′ = OMe), dissolved in acetic acid (0.5 cc.) and oxidized with 0.4 cc. of a 2% chromium trioxide–acetic acid solution at 18° for 12 hours. Isolation with ether gave a solid residue which on crystallization from ether–petroleum ether gave a further 9.5 mg. of (LXXXIV, R = Ac, R′ = OMe), m.p. 148–153° (Kof.) (total yield 27.5 mg., 61%). Further crystallization raised the m.p. to 153–154.5° (Kof.). An authentic sample had m.p. 152.5–154.5° (Kof.),[113] and there was no depression on admixture.

(110) Lardon and Reichstein, footnote 61. A sample was kindly provided by Dr. H. Heymann.

(111) *Cf.* Heymann and Fieser, THIS JOURNAL, **73**, 5252 (1951).

(112) Lardon and Reichstein, (footnote 109) give m.p. 183–185° for this substance.

We wish to express our very warm appreciation to our preparative assistants, Mrs. Dorothy Voitle and Mr. Irving Osvar. Their skill, hard work and enthusiasm were decisive factors for the successful outcome of this investigation. We are indebted to Dr. Ajay K. Bose and Dr. Richard B. Turner for valuable improvements in certain stages of the synthesis.

For their very generous coöperation, we should like to thank the Monsanto Chemical Company, who placed at our disposal large quantities of 4-methoxytoluquinone and the *trans* adduct (IX), and Merck and Company, Inc., who provided various crucial materials.

Finally, we are grateful to Merck and Company, Inc., for their confidence in supporting our program at its outset, and to Merck, Research Corporation, Eli Lilly and Company, and the United States Public Health Service for continued liberal financial support.

(113) *Inter al.*, v. Euw, Lardon and Reichstein (footnote 63) give m.p. 152–153°.

CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Studies on Carcinolytic Compounds. IV. 6-Chloro-9-(1′-glycityl)-isoalloxazines

BY CLIFFORD H. SHUNK, FRANK R. KONIUSZY AND KARL FOLKERS

RECEIVED MARCH 31, 1952

Eleven isoalloxazines in which the substituents in the 6-, 7- and 9-positions were varied have been prepared. Seven 6-chloro-9-glycitylisoalloxazines, two 6-methyl-7-chloro-9-glycitylisoalloxazines and the 6-methyl and the 6-methoxy derivatives of 9-dulcitylisoalloxazine were synthesized by the reaction of alloxan with the diamine obtained by hydrogenation of the appropriately substituted 2-nitro-N-glycitylaniline. The isoalloxazines were tested for their effect in enhancing the rate of regression of lymphosarcoma (6C3H-ED) transplants in C3H mice maintained on a riboflavin deficient diet. 6-Chloro-9-(1′-D-sorbityl)-isoalloxazine appeared to show some activity in several tests. The other compounds showed questionable or negative results in single tests.

6,7-Dichloro-9-(1′-D-sorbityl)-isoalloxazine[1] was found to be effective in enhancing the rate of regression of lymphosarcoma transplants in mice. Addition isoalloxazines in which the substituents in the 6-, 7- and 9-positions are varied have been synthesized. Seven 6-chloro-9-glycitylisoalloxazines, two 6-methyl-7-chloro-9-glycitylisoalloxazines and 6-methyl- and 6-methoxy-9-dulcitylisoalloxazine were prepared.

1-Chloro-4-iodo-3-nitrobenzene[2] was heated in pyridine with D-glucamine,[1] D-galactamine,[1] D-mannamine,[3] L-arabinamine,[1] D-arabinamine,[4] D-ribamine[3] and D-xylamine[4] giving 4-chloro-2-nitro-N-(1′-D-sorbityl)-aniline (I), 4-chloro-2-nitro-N-(1′-D-dulcityl)-aniline (II), 4-chloro-2-nitro-N-(1′-D-mannityl)-aniline (III), 4-chloro-2-nitro-N-(1′-L-arabityl)-aniline (IV), 4-chloro-2-nitro-N-(1′-D-arabityl)-aniline (V), 4-chloro-2-nitro-N-(1′-D-ribityl)-aniline (VI) and 4-chloro-2-nitro-N-(1′-D-xylityl)-aniline (VII), respectively. Reaction of 2-chloro-4-iodo-5-nitrotoluene, prepared by the diazotization of 4-amino-2-chloro-5-nitrotoluene[5] followed by treatment with potassium iodide, with D-glucamine and with D-galactamine yielded 3-chloro-4-methyl-6-nitro-N-(1′-D-sorbityl)-aniline (VIII), and 3-chloro-4-methyl-6-nitro-N-(1′-D-dulcityl)-aniline (IX). 4-Methyl-2-nitro-N-(1′-D-dulcityl)-aniline (X) and 4-methoxy-2-nitro-N-(1′-D-dulcityl)-aniline (XI) were prepared by the reaction of D-galactamine with 4-iodo-3-nitrotoluene[6] and with 4-iodo-3-nitroanisole,[7] respectively.

In the condensation of the substituted iodoben-

(1) F. W. Holly, E. W. Peel, R. Mozingo and K. Folkers, THIS JOURNAL, **72**, 5416 (1950).

(2) Körner, *Gazz. chim. ital.*, **4**, 381 (1874).

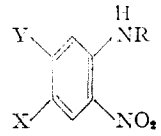
(3) F. W. Holly, E. W. Peel, J. J. Cahill, F. R. Koniuzy and K. Folkers, THIS JOURNAL, **74**, 4047 (1952).

(4) F. W. Holly, E. W. Peel, J. J. Cahill and K. Folkers, *ibid.*, **73**, 332 (1951).

(5) J. Blanksma, *Rec. trav. chim.*, **39**, 410 (1910).

(6) C. Willgerodt and M. Simonis, *Ber.*, **39**, 269 (1906).

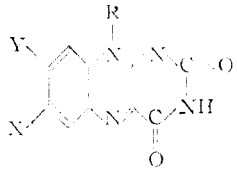
(7) K. Hata, K. Tatematsu and B. Kubata, *Bull. Chem. Soc. Japan*, **10**, 425 (1935).



I, X = Cl, Y = H, R = D-sorbityl
II, X = Cl, Y = H, R = D-dulcityl
III, X = Cl, Y = H, R = D-mannityl
IV, X = Cl, Y = H, R = L-arabityl
V, X = Cl, Y = H, R = D-arabityl
VI, X = Cl, Y = H, R = D-ribityl
VII, X = Cl, Y = H, R = D-xylityl
VIII, X = CH_3, Y = Cl, R = D-sorbityl
IX, X = CH_3, Y = Cl, R = D-dulcityl
X, X = CH_3, Y = H, R = D-dulcityl
XI, X = OCH_3, Y = H, R = D-dulcityl

zenes with the different glycamines, the yields of the substituted anilines varied from 52% for 4-chloro-2-nitro-N-(1′-D-mannityl)-aniline (III) to 0.6% for 4-methoxy-2-nitro-N-(1′-D-dulcityl)-aniline (XI). In the reaction of D-galactamine with the 2-nitroiodobenzenes containing the chloro, the methyl and the methoxy substituents in the 4-positions, the respective yields were 22%, 2.6% and 0.6%. This wide variation may be attributed to the fact that the chloro, the methyl and the methoxy groups when para to the site of nucleophilic displacement stand in the following order of activating power: Cl > CH_3 > OCH_3, the chloro group being the most activating.[8]

Reduction of the 4-chloro-2-nitro-N-glycityl anilines I–VII in aqueous acetic acid over a palladium catalyst followed by reaction of the resulting diamines with alloxan in the presence of boric acid[9] yielded 6-chloro-9-(1′-D-sorbityl)-isoalloxazine (XII), 6-chloro-9-(1′-D-dulcityl)-isoalloxazine (XIII), 6-chloro-9-(1′-D-mannityl)-isoalloxazine (XIV), 6-chloro-9-(1′-L-arabityl)-isoalloxazine (XV), 6-chloro-9-(1′-D-arabityl)-isoalloxazine (XVI), 6-chloro-9-(1′-D-ribityl)-isoalloxazine (XVII) and 6-chloro-9-(1′-D-xylityl)-isoalloxazine (XVIII). Similar treatment of the 3-chloro-4-methyl-6-nitro-N-glycitylanilines VIII and IX yielded 6-methyl-7-chloro-9-(1′-D-sorbityl)-isoalloxazine (XIX) and 6-methyl-7-chloro-9-(1′-D-dulcityl)-isoalloxazine (XX). 6-Methyl-9-(1′-D-dulcityl)-isoalloxazine (XXI) and 6-methoxy-9-(1′-D-dulcityl)-isoalloxazine (XXII) were prepared from the 4-methyl and the 4-methoxy derivatives of 2-nitro-N-(1′-D-dulcityl)-aniline (X) and (X1). The isoalloxazines were obtained in 48 to 70% yield from the corresponding nitroanilines.



XII, X = Cl, Y = H, R = D-sorbityl
XIII, X = Cl, Y = H, R = D-dulcityl
XIV, X = Cl, Y = H, R = D-mannityl
XV, X = Cl, Y = H, R = L-arabityl
XVI, X = Cl, Y = H, R = D-arabityl
XVII, X = Cl, Y = H, R = D-ribityl
XVIII, X = Cl, Y = H, R = D-xylityl
XIX, X = CH_3, Y = Cl, R = D-sorbityl
XX, X = CH_3, Y = Cl, R = D-dulcityl
XXI, X = CH_3, Y = H, R = D-dulcityl
XXII, X = OCH_3, Y = H, R = D-dulcityl

The isoalloxazines were tested by Dr. Gladys A. Emerson of the Merck Institute for Therapeutic Research for their effect in enhancing the rate of regression of lymphosarcoma (6C3H-ED) transplants in C3H mice maintained on a riboflavin deficient diet. 6-Chloro-9-(1′-D-sorbityl)-isoalloxazine (XII) appeared to show some activity in several tests. Preliminary tests indicate that 6-chloro-9-(1′-D-dulcityl)-isoalloxazine (XIII), 6-chloro-9-(1′-L-arabityl)-isoalloxazine (XV) and 6-chloro-9-(1′-D-ribityl)-isoalloxazine (XVII) may possess a low order of activity. The compounds 6-chloro-9-(1′-D-mannityl)-isoalloxazine (XIV), 6-6-chloro-9-(1′-D-arabityl)-isoalloxazine (XVI), 6-chloro-9-(1′-D-xylityl)-isoalloxazine (XVIII), 6-methyl-7-chloro-9-(1′-D-sorbityl)-isoalloxazine (XIX), 6-methyl-7-chloro-9-(1′-D-dulcityl)-isoalloxazine (XX), 6-methyl-9-(1′-D-dulcityl)-isoalloxazine (XXI) and 6-methoxy-9-(1′-D-dulcityl)-isoalloxazine (XXII) were inactive in single tests.

Experimental[10]

Glycamines.—D-Glucamine,[1] D-galactamine,[1] D-mannamine,[3] D-arabinamine,[4] L-arabinamine,[1] D-xylamine[4] and D-ribamine[3] were prepared by the hydrogenation of the corresponding sugars in liquid ammonia.

2-Chloro-4-iodo-5-nitrotoluene.—Twenty grams of 4-amino-2-chloro-5-nitrotoluene[5] was dissolved in 40 ml. of sulfuric acid (sp. gr. 1.84) and the solution was added in a fine stream with stirring to 250 g. of finely chopped ice. The mixture was cooled externally so the temperature was kept at 0° or below. Sodium nitrite (8.4 g.) in 20 ml. of water was added to the resulting slurry over a period of 30 minutes, the temperature being maintained at 0 to −5°. After stirring for an additional ten minutes the solution was filtered. The cold filtrate was added dropwise to a stirred solution of 45 g. of potassium iodide in 200 ml. of water. The temperature of this mixture was kept at 40°. The 2-chloro-4-iodo-5-nitrotoluene that separated was collected, washed with water and dried under reduced pressure, m.p. 64–67°; wt. 29.3 g. Recrystallization from methanol raised the melting point to 70–71°.

Anal. Calcd. for $C_7H_5NO_2ClI$: C, 28.26; H, 1.69. Found: C, 28.56; H, 1.52.

When the 4-amino-2-chloro-5-nitrotoluene was dissolved in sulfuric acid and precipitated by pouring on ice before the diazotization the yield of the 2-chloro-4-iodo-5-nitrotoluene was 92%. In an experiment in which the starting product was finely powdered in a ball mill and then suspended in the diluted sulfuric acid the yield of the iodo compound dropped to 26%. Sixty-nine per cent. of the starting product was recovered.

TABLE I

NITROANILINES

	Compound	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	M.p., °C.
I	$C_{13}H_{17}N_2O_7Cl$	9	42.80	42.97	5.09	4.77	166–167
II	$C_{13}H_{17}N_2O_7Cl$	22	42.80	42.58	5.09	5.36	228–229
III	$C_{13}H_{17}N_2O_7Cl$	52	[a]				168–169
IV	$C_{11}H_{15}N_2O_6Cl$	19	43.08	43.21	4.93	4.85	218–219
V	$C_{11}H_{15}N_2O_6Cl$	6	[a]				215–216
VI	$C_{11}H_{15}N_2O_6Cl$	19	[a]				171–172
VII	$C_{11}H_{15}N_2O_6Cl$	8	[a]				143–145
VIII	$C_{13}H_{19}N_2O_7Cl$	26	44.51	44.85	5.46	5.44	197–198
IX	$C_{13}H_{19}N_2O_7Cl$	15	44.51	44.67	5.46	5.40	256–258
X	$C_{13}H_{20}N_2O_7$	2.6	49.36	49.49	6.37	6.65	210–214
XI	$C_{13}H_{20}N_2O_8$	0.6	46.98	46.50	6.07	5.74	214–215

[a] The compound was not analyzed but was used directly in the next step.

(8) J. F. Bunnett and R. E. Zahler, *Chem. Revs.*, **49**, 273 (1951).

(9) R. Kuhn and F. Weygand, *Ber.*, **68**, 1282 (1935).

(10) Melting points were determined on a Kofler micro hot-stage.

TABLE II

ISOALLOXAZINES

	Compound	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	M.p., °C.
XII	$C_{16}H_{17}N_4O_7Cl$	54	46.55	46.80	4.15	4.22	235–245
XIII	$C_{16}H_{17}N_4O_7Cl$	70	46.55	46.38	4.15	4.14	276–279
XIV	$C_{16}H_{17}N_4O_7Cl\cdot H_2O$	64	44.61	44.79	4.49	4.21	289–290
XV	$C_{15}H_{15}N_4O_6Cl$	66	47.06	47.21	3.95	3.99	271–272
XVI	$C_{15}H_{15}N_4O_6Cl$	67	47.06	47.26	3.95	4.02	271–273
XVII	$C_{15}H_{15}N_4O_6Cl$	56	47.06	47.26	3.95	3.80	263–264
XVIII	$C_{15}H_{15}N_4O_6Cl$	52	47.06	47.35	3.95	3.99	282–283
XIX	$C_{17}H_{19}N_4O_7Cl$	48	47.84	47.54	4.49	4.84	255–256
XX	$C_{17}H_{19}N_4O_7Cl$	66	47.84	47.85	4.49	4.34	276–278
XXI	$C_{17}H_{20}N_4O_7$	65	52.03	52.08	5.14	5.43	285–290
XXII	$C_{17}H_{20}N_4O_8$	51	49.99	49.92	4.94	5.33	277–280

1-Chloro-4-iodo-3-nitrobenzene,[2] 4-iodo-3-nitrotoluene[6] and 4-iodo-3-nitroanisole[7] were prepared from the corresponding 4-amino compounds.

Preparation of Nitroanilines I–XI.—A synthesis of 4-chloro-2-nitro-N-(1′-D-sorbityl)-aniline is reported as representative of the method used.

Twenty grams of D-glucamine (85% pure by titration) and 50 g. of 1-chloro-4-iodo-3-nitrobenzene in 150 ml. of pyridine were heated at reflux temperature with stirring in a nitrogen atmosphere for six hours. Steam was passed into the solution until the pyridine had been removed and the resulting mixture was concentrated under reduced pressure. The residue was triturated with cold water and the solid was collected and washed with acetone giving 4.0 g. of an orange solid, m.p. 148–150°. Recrystallization from methanol gave 2.8 g. of 4-chloro-2-nitro-N-(1′-D-sorbityl)-aniline, m.p. 166–167°.

The nitroanilines are described in Table I.

Preparation of Isoalloxazines XII to XXII.—A synthesis of 6-chloro-9-(1′-D-sorbityl)-isoalloxazine is reported to illustrate the method used.

4-Chloro-2-nitro-N-(1′-D-sorbityl)-aniline (2.7 g.) in 100 ml. of 80% acetic acid was hydrogenated using 1.0 g. of palladium-on-Darco catalyst (5% palladium). After removing the catalyst the filtrate was added to a suspension of 1.5 g. of alloxan and 3.4 g. of boric acid in 110 ml. of acetic acid. After standing at room temperature for two days, the solvent was distilled under reduced pressure and two portions of ethanol were added to the residue and distilled. The residue was triturated with a minimum amount of cold water and the precipitate was collected and recrystallized from water containing about 0.5% acetic acid. A second recrystallization gave 1.8 g. of 6-chloro-9-(1′-D-orbityl)-isoalloxazine, m.p. 235–245° with softening at 210°.

The isoalloxazines are described in Table II.

RAHWAY, NEW JERSEY

[CONTRIBUTION FROM THE DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL]

Isoleucine and Valine Metabolism in *Escherichia coli*. III. A Method for the Quantitative Determination of α-Keto Acid Analogs of Isoleucine and Valine[1]

BY H. EDWIN UMBARGER AND BORIS MAGASANIK

RECEIVED MARCH 17, 1952

A method is described for the separation of mixtures of α-keto acids by paper chromatography and for the quantitative estimation of their components. The method consists of finding the positions of the keto acids by examination under ultraviolet light after treatment of the paper with semicarbazide and subsequent conversion of the semicarbazones to the corresponding 2,4-dinitrophenylhydrazones, which are extracted from the paper with alkali and determined colorimetrically. The accuracy of the method is about ±5%.

The separation and identification of the individual components in a mixture of keto acids by paper chromatography has been described in an earlier communication from this Laboratory.[2] This method proved to be of considerable value in the identification of the α-keto acid analogs of isoleucine and valine accumulating in the culture fluids of a biochemically deficient mutant of *Escherichia coli*[3] and the subsequent demonstration of their role as the immediate precursors of isoleucine and valine in a variety of microörganisms.[4]

The present paper deals with the application of this technique to the quantitative estimation of α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids in mixtures of keto acids. Preliminary experiments indicate that the same method may also be used for the estimation of pyruvic acid and α-ketoglutaric acid. Because of the great importance of α-keto acids in metabolic processes, such a method should be generally useful. Its application to the study of the transamination reactions of isoleucine and valine is described in the following paper.[5]

Experimental

Filter Paper.—Of several commonly used papers which were tested only Eaton and Dikeman (E & D) 613 was sufficiently inert to the semicarbazide treatment to permit observation of the exact location and of the extent of the keto acid spots.

(1) This work was supported in part by funds received from the Eugene Higgins Trust and by a grant from the American Cancer Society to Harvard University. This paper was presented in part before the Division of Biological Chemistry at the 119th Meeting of the American Chemical Society in Boston, Massachusetts, April 4, 1951.

(2) B. Magasanik and H. E. Umbarger, THIS JOURNAL, **72**, 2308 (1950).

(3) H. E. Umbarger and B. Magasanik, *J. Biol. Chem.*, **189**, 287 (1951).

(4) H. E. Umbarger and E. A. Adelberg, *ibid.*, **192**, 883 (1951).

(5) H. E. Umbarger and B. Magasanik, THIS JOURNAL, **74**, 4256 (1952).