George B. Brown

Editor-in-Chief

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PREFACE

The Editors welcome suggestions from the international biochemical community for increasing the usefulness of *Biochemical Preparations* and invite contributions to forthcoming volumes as well as the discussion of potential manuscripts. Manuscripts should be submitted in triplicate in the style currently employed in these volumes. Correspondence may be addressed to the Chairman of the Editorial Board, Dr. Carl S. Vestling, Department of Chemistry, University of Illinois, Urbana, or to the editor of Volume 11, Dr. Sune Bergström, Medical Karoliuska Institutet, Stockholm, Sweden.

It is a pleasure to acknowledge the efforts of those who submitted and those who checked preparations, and those of K.M.B. for editorial assistance and advice.

G.B.B.

August, 1963

COMPOUNDS OF BIOCHEMICAL INTEREST WHICH HAVE APPEARED IN *ORGANIC SYNTHESES* (THROUGH VOLUME 43)

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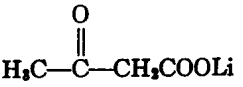
ACETOACETIC ACID (LITHIUM SALT)



Mol. wt 108.03 ($C_4H_5O_3Li$)

Submitted by LEO M. HALL, Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville.
Checked by GLORIA T. SCOTTO and GEORGE B. BROWN, Sloan-Kettering Institute for Cancer Research, New York.

I. Principle

Ethyl acetoacetate is hydrolyzed in the presence of LiOH. The product is crystallized as the lithium salt from methanol-ether.

II. Starting Materials

Ethyl acetoacetate, a commercial product, is redistilled under reduced pressure (b.p. 76–80°/18 mm.) before use.

LiOH, 4.00*M* solution in water; absolute methanol; absolute ethyl ether.

III. Procedure

A mixture of 5.2 g. (0.04 mole) of ethyl acetoacetate, 25 ml. of water, and 10.0 ml. of 4.00*M* LiOH is placed in a water bath at 40° for 4 hours. The pale yellow solution is evaporated to dryness under reduced pressure at a bath temperature of 40°.[1] The dry crystalline residue is dissolved in three successive 15-ml. portions of absolute methanol, and insoluble material is removed by suction filtration through S & S No. 576 filter paper.[2] Absolute ethyl ether, about 25–50

[1] It is essential that the flask contents be completely dry, or crystallization from methanol-ether will be difficult. The use of a rotating evaporator is advantageous. The checkers dried the residue over P_2O_5 in vacuum overnight.

[2] Difficulty may be experienced in obtaining a clear filtrate. Centrifugation after addition of 0.2 g. of Hyflo Super Cel (Fisher Scientific Co.) will clarify the solution.

ml., is added slowly until crystallization begins. After 30 minutes at room temperature, ether is added slowly until a total of 225 ml. is reached, and the solution is cooled for 3–4 hours at 4°. The crystals are collected by suction filtration, washed with anhydrous ether, and dried in vacuum. The product is recrystallized twice by dissolving the crystals in 6 ml. of absolute methanol per gram. Insoluble material is removed by filtration or centrifugation.[2] Absolute ether is added as before, and the crystals are collected and dried in vacuum. After two recrystallizations, the product is pure, and the yield, based on the ethyl acetoacetate, is between 70% and 80%.[3] If the sodium salt is desired, the compound is dissolved before use in a minimum amount of water and passed through a column containing a threefold excess of Dowex 50 (Na^+, 200–400 mesh) at 0–4°. Recovery from the column is quantitative.

IV. Properties and Purity of Product

The crystalline lithium salt is non-hydroscopic and stable for at least several months when kept under vacuum in the cold. *Analyses*: Calcd. for $C_4H_5O_3Li$: C, 44.47; H, 4.67; Li, 6.42. Found: C, 44.52; H, 4.84; Li, 6.39. Decomposition of the compound with aniline citrate yielded 99.93% of the calculated amount of CO_2.[4] Neutral solutions of either the lithium or the sodium salt may be kept frozen at −15° for 2–3 weeks without significant decomposition.

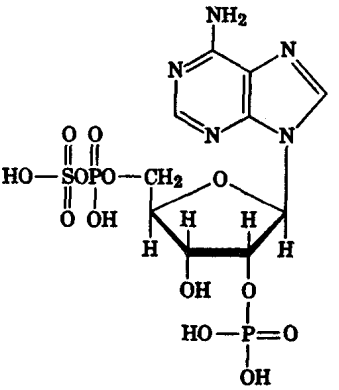
[3] The checkers obtained 10% to 20% lower yields. Traces of water in the methanol, or high humidity, is responsible for decreased yields.

[4] Aniline citrate catalyzed CO_2 evolution for acetoacetate was measured manometrically at 15° (P. P. Cohen, in *Manometric Techniques*, Umbreit, Burris, and Stauffer, eds., p. 211, Burgess, Minneapolis) or by the following modification of the titrametric micromethod of West, Christensen, and Rinehart [E. S. West, B. E. Christensen, and R. E. Rinehart, *J. Biol. Chem.*, **132**, 681 (1940)]: Two Erlenmeyer flasks, 10 and 25 ml. capacity, were joined by a 4-cm. glass side arm. The weighed sample was introduced into the smaller flask, and the larger was charged with a standardized solution of $Ba(OH)_2$. Both flasks were quickly stoppered and evacuated to 20 mm. Hg through a hypodermic needle inserted through one of the stoppers. The evacuation needle was removed, and 3.0 ml. of aniline citrate reagent (see the preceding reference) was introduced into the sample flask with needle and syringe. After the sample flask had been warmed at 40° for 3 hours, evolution and diffusion of CO_2 was complete. CO_2-free air was let into the flasks with a needle, and excess $Ba(OH)_2$ was titrated with standardized HCl. Duplicate determinations with suitable blanks agreed within 0.2%. Both procedures gave essentially identical results, but the manometric method was considerably less precise.

V. Methods of Preparation

Acetoacetic acid has usually been prepared in dilute solution by hydrolysis of the ethyl ester with a slight excess of NaOH.[5,6] The crystallization of acetoacetic acid has been reported.[7]

ADENOSINE 3′-PHOSPHATE 5′-SULFATOPHOSPHATE, "ACTIVE SULFATE"



Submitted by JAMES BADDILEY and ARNOLD R. SANDERSON, King's College, University of Durham, Newcastle upon Tyne, England.
Checked by J. G. MOFFATT, British Columbia Research Council, Vancouver.

I. Principle

The pyridine-sulfur trioxide complex reacts with adenosine 3′:5′-diphosphate in $NaHCO_3$ solution to give a mixture which contains adenosine 2′:3′(hydrogen)-phosphate 5′-phosphate and adenosine 3′-phosphate 5′-sulfatophosphate. The sulfatophosphate is isolated by charcoal adsorption and anion-exchange chromatography under neutral conditions.

II. Procedure

Paper chromatography. Chromatography is carried out on Whatman No. 4 paper, which has previously been washed with dilute acetic

[5] R. Davies, *Biochem. J.*, **37**, 230 (1943).
[6] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **39**, 408 (1945).
[7] R. C. Krueger, *J. Am. Chem. Soc.*, **74**, 5536 (1952).

acid and water. The following solvent systems are used: (*A*) saturated ammonium sulfate-0.1*M*-ammonium acetate(*p*H 6.0)-isopropanol (17:19:2);[1] (*B*) *n*-propanol-ammonia(*d* 0.88)-water (6:3:1). Nucleotides are located by inspection under ultraviolet light.

Adenosine 3′-phosphate 5′-sulfatophosphate. The pyridine-sulfur trioxide complex (pyridine-*N*-sulfonic acid)[2] (800 mg.) is added, with stirring, to a solution of the lithium salt of adenosine 3′:5′-diphosphate[3-5] (200 mg.) and $NaHCO_3$ (1.2 g.) in water (12 ml.) at 45°. After 40 minutes at this temperature, the solution is cooled, diluted with iced water (500 ml.), adjusted to *p*H 6 with 1*N* formic acid, and passed through a column (7 x 5 cm.) of Norit A charcoal/Supercel silica (4:3), at a rate of 4 ml./minute. The charcoal is previously washed with *N* HCl, *N* NH_4OH, water, ethanol, and ether, then dried at 110° for 16 hours. The column is washed until the washings are free of sulfate and their optical density at 257.5 mμ is less than 0.1.

Nucleotides are eluted (1.5 ml. per minute) from the column with 50% aqueous ethanol which contains 1% of *N* NH_4OH. The optical density (257.5 mμ) of the eluate indicates a recovery of 87%. The eluate is then passed through a column (7 x 4 cm.) of Dowex 1 (x2) resin (chloride form; 200–400 mesh) which is washed with water (3 liters). Concave gradient elution is performed with use of 2*M* LiCl solution (2 liters) as eluent in the reservoir, and water (3 liters) in the mixing flask; the system is similar to that previously described.[6] Fractions (25 ml.) are collected at a rate of 10 ml. per minute and the optical density at 257.5 mμ of each fraction is measured. Two sharp peaks are observed. One peak corresponds to adenosine 3′:5′-diphosphate containing a trace of adenosine 2′:3′ hydrogen phosphate 5′-phosphate, while the second corresponds to adenosine 3′-phosphate 5′-sulfatophosphate. The yield of sulfatophosphate, calculated from ultraviolet measurements, is about 10%. The fractions containing the desired nucleotide are passed through a Norit A charcoal/Supercel column (2 x 5 cm.), similar to that mentioned previously, at a rate of 10 ml. per minute. After being washed with water (2 liters), the

[1] R. Markham and J. D. Smith, *Biochem. J.*, **49**, 401 (1951).
[2] P. Baumgarten, *Ber.*, **59**, 1166 (1926).
[3] F. Cramer, G. W. Kenner, N. A. Hughes, and A. Todd, *J. Chem. Soc.*, **1957**, 3297.
[4] J. Baddiley, J. G. Buchanan, and R. Letters, *J. Chem. Soc.*, **1958**, 1000.
[5] J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).
[6] J. Baddiley, J. G. Buchanan, R. Letters, and A. R. Sanderson, *J. Chem. Soc.*, **1959**, 1731.

nucleotides are eluted with 50% aqueous ethanol (300 ml.) which contains 1% of *N* NH_4OH. The optical density of the eluate at 257.5 mμ indicates a recovery of 88%. The eluate is concentrated under reduced pressure below 35° and passed through a column (1 x 1 cm.) of Dowex 50 (x8) resin (lithium form; 200–400 mesh). Evaporation of the eluate and washings yields the lithium salt of active sulfate (19 mg.).

III. Properties and Purity of Product

A sample of the product is almost homogeneous when examined by paper chromatography and paper electrophoresis; about 5% of adenosine 3′:5′-diphosphate is present. The diphosphate content slowly increases on storage. The ratios adenosine:P:ribose:S:N (total):N (ammonium) are 1.00:2.02:0.96:0.96:8.95:3.95. These values are determined on a sample of lithium salt (5 mg.) purified by elution of the appropriate ultraviolet-absorbing area from a chromatogram developed in solvent system *B*. The calculated ratios for the tetraammonium salt ($C_{10}H_{27}O_{13}N_9P_2S$) are 1:2:1:1:9:4, respectively.

Active sulfate is partially hydrolyzed by heating in 0.1*N* NaOH for 2 hours at 100°. A sample of the resulting diphosphate may be isolated from the paper after chromatography in solvent system *B*, and, on rechromatography in solvent system *A*, is found to consist solely of adenosine 3′:5′-diphosphate; no trace of the 2′:5′-isomer is detected.

IV. Methods of Preparation

Adenosine 3′-phosphate 5′-sulfatophosphate is an intermediate in the biosynthesis of sulfuric esters.[7-9] It is formed enzymatically from adenosine triphosphate and inorganic sulfate. It has been synthesized by a method similar to that described here.[6]

[7] H. Hilz and F. Lipmann, *Proc. Natl. Acad. Sci., U. S.*, **41**, 888 (1955).

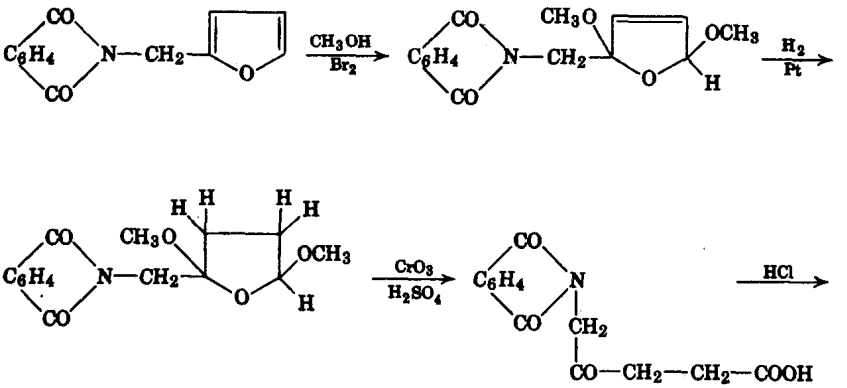
[8] P. W. Robbins and F. Lipmann, *J. Biol. Chem.*, **229**, 837 (1957); **233**, 681, 686 (1958).

[9] L. G. Wilson and R. S. Bandurski, *Arch. Biochem. Biophys.*, **62**, 503 (1956); *J. Biol. Chem.*, **233**, 975 (1958).

δ-AMINOLEVULINIC ACID

Submitted by FABIO SPARATORE[1] and WILLIAM CUMMING,
The Rockefeller Institute, New York.
Checked by BARRY NORTH and DAVID SHEMIN.
Columbia University, New York.

I. Principle



$$H_2N—CH_2—CO—CH_2—CH_2—COOH\cdot HCl$$

II. Procedure

A. *cis*- and *trans*- 2-Phthalimidomethyl-2, 5-dimethoxytetrahydrofuran. (The first part of this preparation should be carried out in a hood.) *N*-Phthaloylfurfurylamine[2] (56.5 g., 0.25 mole) is dissolved in

[1] Present address, Chemistry Department, University of Genoa. This investigation was supported by a research grant to Dr. S. Granick from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, RG-4922.

[2] Finely powdered phthalic anhydride (75 g., 0.5 mole) is added to 50 g. (0.5 mole) of commercial furfurylamine with thorough mixing and cooling. The flask is then connected to a short air condenser and immersed for 15–20 minutes in an oil bath kept at 140–145°. The cooled dark product is crystallized from about 600 ml. of boiling methanol. The crystals are collected, and the mother liquor is concentrated to a small volume to give a second crop of *N*-phthaloylfurfurylamine. The total yield is 106 g. (90% yield) of almost colorless crystals, melting at 120–121°.

warm methanol (1.5 liters), a methanolic solution of dry potassium acetate (50 g. in 200 ml.) is added, and the mixture is cooled to about 30–35°. To this solution a cold solution of bromine (12.5 ml., 0.25 mole) in methanol (75 ml.) is added dropwise from a buret during 1.5 hours [3] and with efficient stirring. The solution is stirred again for 1.5 hours after completion of the addition of bromine. The solution is then concentrated under reduced pressure to about 700 ml.,[4] the precipitated potassium bromide is removed by filtration, and the solution is hydrogenated at atmospheric pressure, with platinum dioxide [5] (1.5 g.) as catalyst, in one or several portions depending on the capacity of the hydrogenation vessel. The hydrogenation is continued until about 5 liters of hydrogen (0° and 760 mm.) is absorbed. Theory, 5.6 liters.

The catalyst is removed and the solution evaporated to dryness under reduced pressure at about 35°. The residue is triturated with a little cold water, filtered, and dried over KOH in vacuum.[6] The weight of dried material is 60–66 g.

B. 5-(Phthaloyl)-aminolevulinic acid. A solution of chromium trioxide (24 g., 0.24 mole) in water (120 ml.) and concentrated H_2SO_4 (18 ml.) are added to a stirred solution of the above crude mixture of *cis* and *trans* isomers (15 g., 0.051 mole) in acetone (160 ml.) at such a rate that the temperature remains between 16° and 20° (about 2 hours).[7]

The solution is stirred for two additional hours at about 20°, diluted with water (100–150 ml.), and the acetone removed under reduced pressure. The precipitated 5-(phthaloyl)-aminolevulinic acid is removed by filtration and washed thoroughly with cold water to elim-

[3] A small precipitate of *N*-phthaloylfurfurylamine dissolves as the reaction proceeds and the temperature can be decreased to about 20°. After a certain time KBr begins to separate.

[4] Concentration to a smaller volume causes precipitation of organic material in addition to KBr. This may be checked by noting the solubility of the precipitate in a little water. Any organic material may be recovered by dissolving the precipitate in water, collecting the insoluble material on a filter, and redissolving in methanol.

[5] The catalyst was obtained from Baker & Co., Inc., Newark, New Jersey, but it could be prepared as described in *Org. Syntheses Coll. Vol.* **1**, 463 (1941). Palladium over charcoal (5% Pd) (8 g.) can also be used, but the hydrogen absorption is much slower.

[6] This is convenient in order to calculate the approximate amount of oxidant for the next step.

[7] The rate of addition of the oxidant and the amount of reactants used depend on the efficiency of the cooling. The rate and amounts specified here allow easy control with cold water.

inate the chromium salt. The mother liquors are extracted several times with a mixture of benzene and ether (equal volumes) and the solvents evaporated in vacuum. The oily residue is redissolved in a very small volume of methanol and left in the cold; after long standing a second crop of 5-(phthaloyl)-aminolevulinic acid is obtained. This product can be recrystallized from methanol and water or acetone and water.

Four such oxidations gave 40–45 g. (61–69% yield based on *N*-phthaloylfurfurylamine) of white crystals melting at 162–164° (sintered at 159°).

C. 5-Aminolevulinic acid hydrochloride. The 5-(phthaloyl)-aminolevulinic acid is heated under reflux for 6 hours with 20–30 times its weight of 6*N* hydrochloric acid and cooled. The precipitated phthalic acid is removed by filtration and washed with water. The acid solution is diluted with the same volume of water, extracted four times with ethyl acetate (which in turn is back-extracted with a little water),[8] and the aqueous solution is finally evaporated almost to dryness under reduced pressure at about 40°. The residue is further dried in a vacuum desiccator and then crystallized by solution in cold absolute ethanol and precipitation with dry ether.[9] The yield of the hydrolysis is 91%.

III. Properties and Purity of Product

The melting point may vary from 144° to 151°. *Analysis*: Calcd. for $C_5H_9NO_3 \cdot HCl$ (mol. wt. 167.5), C, 35.84%; H, 6.01%; N, 8.36%. Found: C, 35.97%; H, 6.04%; N, 8.24%. Colorimetric analysis, by the acetylacetone method of Mauzerall and Granick,[10] and comparison of this preparation with a known sample of δ-aminolevulinic acid [11–13] gave the following results: known, $\epsilon_{552m\mu} = 6.84 \pm 0.1 \times 10^4$; this preparation, $\epsilon_{552m\mu} = 6.79 \pm 0.1 \times 10^4$. Maxima for the colored compound occurred at 552 mμ with a shoulder at 525 mμ. The ratio 525 mμ/552 mμ was 0.67 in both samples.

[8] If the 5-(phthaloyl)-aminolevulinic acid is carefully recrystallized before the hydrolysis, the acid solution is practically colorless; if not, it should be decolorized with charcoal.

[9] This operation should be carried out as fast as possible because long standing in ethanol produces some ethyl ester.

[10] D. Mauzerall and S. Granick, *J. Biol. Chem.*, **219**, 435 (1956).

[11] D. Shemin and C. S. Russel, *J. Biol. Chem.*, **215**, 613 (1955).

[12] V. M. Radionov and M. A. Gubareva, *J. Gen. Chem. USSR*, **23**, 1951 (1953); *Chem. Abstr.*, **49**, 1007i (1955).

[13] A. Neuberger and J. J. Scott, *J. Chem. Soc.*, **1954**, 1820.

IV. Methods of Preparation

5-Aminolevulinic acid hydrochloride has been prepared by a combination of the Arndt-Eistert and Gabriel reactions on succinyl chloride monomethyl ester (β-carbomethoxypropionyl chloride).[11-13]

Another method consists of the nitrosation and reduction of β-ketoadipic acid prepared by the condensation of succinyl chloride monomethyl ester with malonic ester.[11,14]

The two methods above have been used to prepare 5-aminolevulinic acid labeled with C^{14} in various positions.[15]

The procedure described here is a modification of the Marei and Raphael synthesis.[16] These authors used *N*-benzoylfurfurylamine, however.

Other methods of preparation have been described.[11,13,17,18]

[14] A. Neuberger and J. J. Scott, *Biochem. J.*, **64**, 137 (1956).

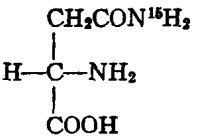
[15] D. Shemin in Colowick and Kaplan, *Methods in Enzymology*, Vol. IV, p. 648, Academic Press, 1957.

[16] A. A. Marei and R. A. Raphael, *J. Chem. Soc.*, 1958, 2624.

[17] R. W. Wynn and A. H. Corwin, *J. Org. Chem.*, **15**, 203 (1950).

[18] A. W. Schrecker and M. M. Trail, *J. Am. Chem. Soc.*, **80**, 6077 (1958).

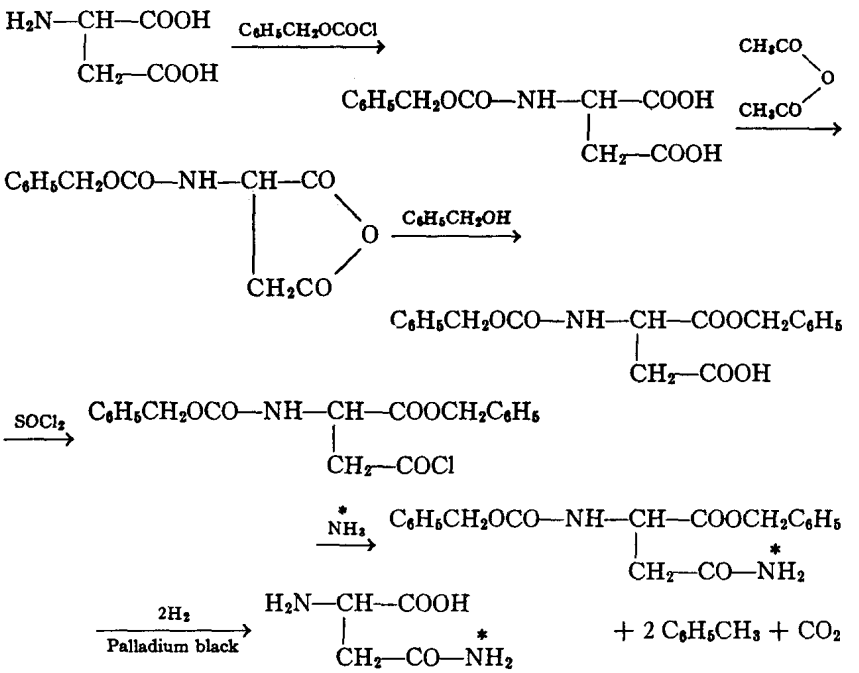
L-ASPARAGINE-N^{15}



Mol. wt. 132.12($C_4H_8O_3N_2$)

Submitted by YUKIO YAMAMOTO, Nagoya University, Japan.
Checked by CHARLOTTE RESSLER, HARRIET RATZKIN, and HILDA MALODECZKY, Institute for Muscle Disease, Inc., New York.

I. Principle



II. Starting Material

The L-aspartic acid, $[\alpha]_D^{20}$ = about 25° (*c* 2, 5*N* HCl), may be obtained from commercial sources and used without further purification, or it may be prepared by the procedure of Vickery and Pucher.[1]

[1] H. B. Vickery and G. W. Pucher, *Biochem. Prepns.*, **2**, 71 (1952).