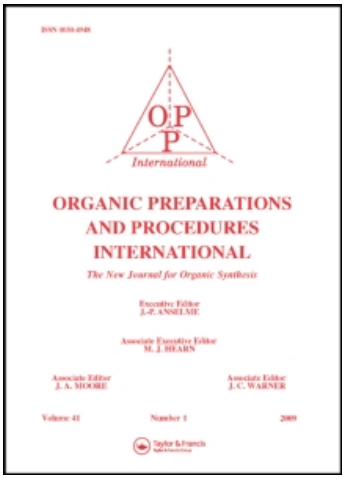

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SYNTHESIS OF PURPURATE-1,1'-DIACETIC ACID (PDAA) TRIPOTASSIUM SALT. A NEW CALCIUM INDICATOR FOR BIOLOGICAL APPLICATIONS

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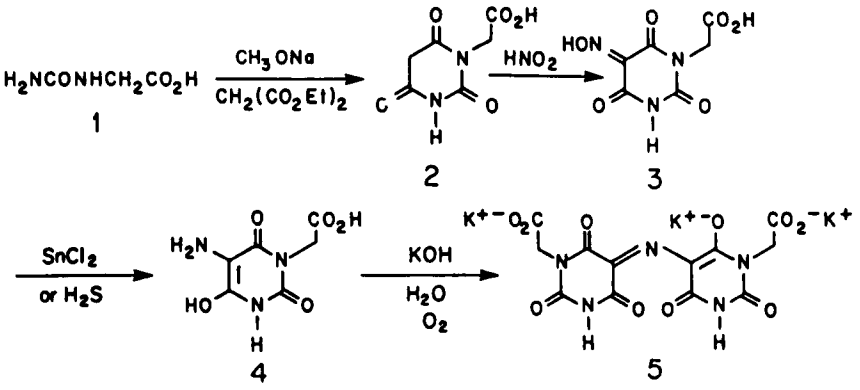
In a previous report[1] we described the application of a new calcium indicator, the triammonium salt of 1,1-dimethylpurpurate-3,3'-diacetic acid (DMPDAA), to the study of calcium transients occurring during muscle contraction. DMPDAA displayed the favorable characteristics observed earlier with tetramethylmurexide (TMX) in this type of application[2] and, in addition, yielded experimental results which were free from the uncertainties of interpretation which had resulted from the permeability of membranes to TMX. However, because the preparation and purification of DMPDAA had proved somewhat difficult, and because, like TMX, it was to some extent bound or sequestered by intracellular constituents, work on the synthesis of other compounds of the murexide (purpurate) type was continued in order to obtain calcium indicators more easily accessible in pure form than is DMPDAA and less prone to attach to structures within cells. The result of this effort was the preparation of purpurate-1,1'-diacetic acid (PDAA) as the tripotassium salt (5). This most recent of the murexide-related calcium indicators has shown a significant improvement over DMPDAA in its suitability for use in muscle contraction studies.

The several-step synthesis required to obtain 5 is detailed below.



The two methyl groups of DMPDAA were omitted from the structure of 5 in the expectation that a less lipophilic molecule would interact less with membranes and hydrophobic regions of proteins. This objective has been approached through a synthetic scheme that begins with the condensation of diethyl malonate with the sodium salt of hydantoic acid (1) in the presence of sodium methoxide to obtain a disodium salt of 1-carboxymethylbarbituric acid (2). Although sodium hydantoate had only a slight solubility in the reaction medium (methanol), the barbituric acid derivative (2) was obtained in quite good yield. Compound (2) was obtained in the free acid form as a crystalline solid following acidification of the disodium salt. The properties of the free acid indicate that two or more tautomers are present; melting points are broad and somewhat variable, and quite different ultraviolet spectra are obtained when the solvent is changed or the pH of aqueous solutions is varied. Neither the highly water-soluble compound 2 nor its salts appear to have been described in the literature previously, although several of its esters had been obtained in unspecified yield by treatment of hydantoic acid esters with malonic acid and phosphorus oxychloride.[4]

Treatment of 2 in water with sodium nitrite produced 1-carboxymethylvioluric acid (3), which precipitated as the crystalline sodium salt

when the reaction was carried out in concentrated solutions. Acidification of the sodium salt afforded 3 in the free acid form, another water-soluble solid. Ultraviolet spectra of 3 also changed with changes in the pH of its aqueous solutions, but samples of 3 showed a rather well-defined melting point. It was found that 3 could be reduced to uramil-1-acetic acid (4) by hydrogen sulfide, sodium dithionite, or, most conveniently, by stannous chloride. Acid 4 is a high-melting crystalline solid, which, in contrast to 2 and 3, has only limited solubility in organic solvents or neutral water. It dissolves readily only in alkaline solutions.

Although autooxidation of uramil derivatives to murexides has been known for a century and a half,[5] it has not been a generally favored procedure for purpurate preparation, and when autooxidation was conducted in aqueous potassium hydroxide, uramil itself yielded precipitates containing some potassium purpurate but consisting mainly of other products. However, acid 4 undergoes a very smooth conversion into the tripotassium salt of purpurate-1,1'-diacetic acid (PDAA) (5) when its solutions in aqueous potassium hydroxide (1.5 equiv. of KOH) are stirred at *ca.* 25° C in contact with air. In a typical experiment in which the progress of reaction was monitored by following the rapidly changing ultraviolet-visible spectrum, formation of the dye was essentially complete after four hours, by which time no spectroscopic evidence of starting material or by-products could be seen. Addition of one or more volumes of absolute ethanol to the aqueous reaction mixture precipitated the product as a purple-black solid, which was collected by centrifugation and purified by a reprecipitation from water solution by addition of ethanol. A 48% yield was obtained of a product showing a molar absorptivity of 15,000 at 524 nm and the elemental composition ($C_{12}H_6K_3N_5O_{10}$), corresponding to 5. The ^{13}C NMR spectrum of the PDAA anion showed six different ^{13}C signals,

corresponding to a symmetrical charge-delocalized resonance-stabilized molecule in which 5 would be one of two equivalent contributing forms. The visible absorption maximum is shifted from 524 to 486 nm when 5 binds calcium ion; the dissociation constant for the calcium complex in a neutral buffered aqueous solution containing 150 mM potassium chloride was 0.95 mM. Results of a study of calcium transients involved in muscle contraction have indicated that 5 is better suited to this purpose than any other currently available indicator.[3] It binds less to cellular constituents than DMPDAA does, is very soluble in water, and causes no evident interference with the the processes involved in muscle contraction.[3]

EXPERIMENTAL SECTION

Microanalyses are by Atlantic Microlab, Inc., Norcross, Georgia. Spectra were determined with the following instruments: UV-VIS spectra on a Hewlett Packard 8452 diode array spectrophotometer; IR spectra on a Nicholet Model 5DXB FT-IR spectrophotometer; NMR spectra on a IBM NR/300 FTNMR spectrometer.

1-Carboxymethylbarbituric Acid (2).- Hydantoic acid (1) (5.9 g., 50 mmol) (Sigma) and diethyl malonate (16 g, 100 mmol) were dissolved in 100 ml of methanol in a reflux apparatus provided with a magnetic stirrer and protected from moisture with a drying tube. Sodium methoxide (8.1 g, 150 mmol) in 34.3 ml of methanol (Aldrich, 25% solution) was added, and the mixture was stirred and refluxed overnight. The white precipitate, the disodium salt of 1-carboxymethylbarbituric acid (2), was filtered from the mixture and washed on the filter with 30 ml of methanol. The yield was 8.0 to 10.5 g (70-90%) of crude product which showed a molar absorptivity of 13,000-18,000 at 256 nm. To obtain 2 as the free acid the disodium salt was dissolved in water and the solution was acidified by addition of slightly more than 2 equivalents of hydrochloric acid. The resulting solution was taken to dryness in vacuo and the residue was extracted with warm acetone. Sodium chloride was removed by filtration, and the acetone filtrate was

allowed to evaporate from an open dish to leave the product as a clear glass or a white crystalline powder. Addition of ethyl acetate facilitated crystallization and yielded a suspension from which 2 was collected by filtration and dried; it melted over the range *ca.* 160-170°.

IR (Nujol): 3655, 3210, 3160, 3100, 1725, 1690, 1440, 1405, 1390, 1350, 1250, 1210, 1160, 1095, 922, 846, 756, 692, 673, 641 cm^{-1}. UV-λ_{max} (log ε) (water, unbuffered) 220 (3.69), 260 (3.58); (water, pH 10) 260 (4.25); (water, pH 1) 214 (3.87), 256 (3.03); (ethanol) 216 (3.59), 258 nm (3.96).

Anal. Calcd. for $C_6H_6N_2O_5$: C, 38.72 H, 3.25 N, 15.05

Found: C, 38.49 H, 3.32 N, 14.99

1-Carboxymethylvioluric Acid (3).- A solution of 2.30 g (10 mmol) of the disodium salt of 2 dissolved in 10 ml of 3N hydrochloric acid was added dropwise to a stirred solution of 1.03 g (15 mmol) of sodium nitrite in 7.5 ml of water in a beaker placed in an ice bath. During the addition the monosodium salt of 3 began to separate as a white or pink crystalline precipitate. The mixture was stirred for 1 hr after the addition was completed, then filtered to collect the precipitated product. The yield was 1.28 g (63%). To obtain the free acid 3 from this sodium salt a 1.275-g quantity was treated with 5 ml of 2 N hydrochloric acid, and the resulting solution was evaporated to dryness in vacuo. The residue was extracted with 15 ml of acetone, and the acetone extract was filtered to remove sodium chloride and allowed to evaporate from an open dish. The residue, a partially crystalline solid, became entirely crystalline when treated with a small volume of ethyl acetate, to give 0.97 g (90% recovery) of white crystals, m.p. 195°. Recrystallization from an acetone-methylene chloride mixture afforded a product melting at 210-212° (dec), after gradual browning and shrinking from *ca.* 170°.

IR (Nujol): 3310, 3240, 1740, 1695, 1450, 1370, 1310, 1295, 1260, 1230,

1145, 1095, 1070, 1015, 966, 867, 785, 720, 629 cm^{-1}. UV-λ_{max} (log ε) (water, pH 1) 252 (4.16); (water, pH 10) 210 (4.19), 312 nm (4.23).

Anal. Calcd. for $C_6H_5N_3O_6$: C, 33.50 H, 2.34 N, 19.54

Found: C, 33.56 H, 2.39 N, 19.43

Uramil-1-acetic Acid (4).- Two methods were developed for reduction of 1-carboxymethylvioluric acid (3) to uramil-1-acetic acid (4). Yields on the order of 70% were obtained by use either of hydrogen sulfide (method A) or of stannous chloride (method B). Since it has not been established which of these methods could be more effectively optimized, both are given here.

Method A.- A 1.9-g quantity (8.0 mmol) of the sodium salt of 3 was suspended in 20 ml of water in a 40-ml centrifuge tube and concentrated hydrohloric acid was added dropwise (several drops needed) until solution of 3 was complete. A brisk stream of hydrogen sulfide was passed into the solution for 1 hr while solid precipitates were separating and collecting mainly on the glass surfaces. These insoluble products were resuspended in the aqueous hydrogen sulfide solution and the mixture was stirred overnight, then filtered to collect the resulting product mixture (1.517 g after drying) consisting of 4 mixed with elemental sulfur. This crude product was suspended in 100 ml of toluene and the suspension was boiled under reflux for 3 hrs. The undissolved solid was collected by filtration to yield 1.11 g (69%) of 4, which darkens and decomposes to a dark viscous melt near 260°. (Evaporation of the toluene filtrate left yellow needles of pure sulfur.) Final purification of an analytical sample was performed by dissolving 4 in dilute aqueous potassium bicarbonate and precipitating the product in crystalline form by acidification with hydrochloric acid.

IR (Nujol): 3065, 1730, 1695, 1615, 1525, 1460, 1400, 1295, 1230, 1185, 1130, 941, 785 cm^{-1}. UV-λ_{max} (log ε) (water) 256 nm (4.29).

^{1}H-NMR (D_2O): δ 4.32 ppm (s, 2H, methylene of carboxymethyl).

Anal. Calcd. for $C_6H_7N_3O_5$: C, 35.82; H, 3.51; N, 20.90

Found: C, 35.53; H, 3.54; N, 20.71

Method B.- The sodium salt of 3 (2.37 g, 10.0 mmol) was suspended in 20 ml of water and dissolved by addition of a few drops of concentrated hydrochloric acid. A solution of stannous choride dihydrate (4.98 g, 20 mmol) in 5.0 ml of concentrated hydrochloric acid was then added in portions with stirring. The mixture was stirred for 30 min, then filtered to collect the product, which was washed on the filter with water (10 ml) and then with acetone. The yield was 1.44 g (70%).

Purpurate-1,1'-Diacetic Acid Tripotassium Salt (5).- A solution of 4 was prepared in an uncovered 50-mm crystallizing dish by adding potassium methoxide (0.210 g, 3.0 mmol) to a suspension of 4 (0.402 g, 2.0 mmol) in 4.0 ml of water. The solution was placed under a small bell jar through which a stream of air was admitted after it had been passed through a drying tube packed with sodium hydroxide pellets used to remove atmospheric carbon dioxide. The solution, which was stirred with a magnetic stirring bar, rapidly developed the purple murexide color when exposed to oxygen in this manner. Spectra taken of samples withdrawn from the solution at intervals showed that conversion to the purpurate 5 was essentially complete at the end of a 4-hr period of stirring in air at *ca.* 25° C. The solution was then transferred to a 40-ml centrifuge tube and diluted with 30 ml of absolute ethanol to separate the product, which was brought down by centrifugation as a purple-black precipitate after the mixture had been allowed to stand for 1 hr. The supernatant was decanted and the product was suspended and stirred in 15 ml of methanol, then collected by centrifugation, washed with acetone and dried in a vacuum desiccator. The yield of crude product was 0.44 g (88%). The dye was purified by

reprecipitation. It was dissolved in 8 ml of water and traces of suspended impurities were separated by centrifugation and decantation of the resulting solution, from which the product was precipitated by dilution with 16 ml of absolute ethanol. The mixture was centrifuged after separation of product appeared complete, and after decantation of the supernatant, the precipiate was washed with 15 ml of methanol, then with 30 ml of acetone and dried in a vacuum desiccator. The recovery of purified product was 0.24 g (48% yield).

UV-VIS: λ_{max}(log ε) (water) 252 (4.22), 324 (3.95), 524 nm (4.18).

^{1}H-NMR (D_2O): δ 4.41 ppm (s, 4 H, methylene of carboxymethyl). ^{13}C-NMR (D_2O): δ 44.4, 120.4, 151.5, 159.1, 160.3, 175.1 ppm.

Anal. Calcd. for $C_{12}H_6K_3N_5O_{10}$: C, 28.97 H, 1.22 N, 14.08

Found: C, 29.05 H, 1.28 N, 13.83

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