The Synthesis of XTT: A New Tetrazolium Reagent that is Bioreducible to a Water-Soluble Formazan

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A new tetrazolium salt, XTT, has been synthesized. XTT is reduced by a considerable variety of cell lines to a water-soluble formazan. XTT appears to merit further investigation as a reagent for broader application to cell culture assay systems.

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This report describes a practical preparative scale synthesis of the tetrazolium salt, sodium, 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro)benzenesulfonic acid hydrate, "XTT" (**1**). This tetrazolium derivative is currently under evaluation in a cell culture assay system being considered for use in the National Cancer Institute's new *in vitro* cultured human tumor cell line anticancer screening program [1a-b, 2a-b].

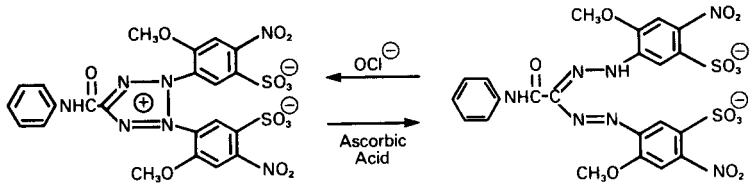
Reviews of tetrazolium salts and formazans are available [3,4]. Many relatively colorless, water-soluble tetrazolium salts which are bioreducible to highly colored, water-insoluble formazans are well known, and several are commercially available. The water solubility of tetrazolium salts is usually attributable to the ionization of the tetrazolium ring. This charge is lost when the ring is opened by reduction leading to uncharged, water-insoluble formazans. The formazans obtained from commercially available tetrazolium salts are usually uncharged and very insoluble in water.

Bioassay systems which rely on the reduction of tetrazolium salts to insoluble formazan products include the histochemical localization of enzyme activities [3,5] and the identification of viable colonies of mammalian cells in soft agar culture [6]. These examples depend on the deposition of the colored formazan at the reaction site. If a tetrazolium salt assay system is used to identify *where* a bioreduction occurs, then the insolubility of the neutral formazan is an advantage because the colored formazan production is localized at the site of reduction.

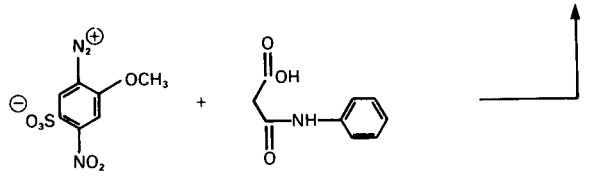
Tetrazolium assays may be used to measure the effects of added substances such as drugs, hormones, or toxins on the growth of cells in culture. These effects can be correlated with the *amount* of formazan produced by the cells under appropriate assay conditions [7,8,9,10]. Also, the ability of added substances to protect cells from various threats such as radiation or attack by viruses can be correlated with the amount of formazan produced in a tetrazolium assay.

If the tetrazolium assay is intended to measure the effect of added substances on cells by measuring the amount of formazan produced, then the localization afforded by an insoluble formazan may or may not be an advantage. For example, the steps needed to dissolve the formazan so that its concentration can be measured spectrophotometrically may involve exposure of personnel to potentially hazardous solutions of toxins or infectious agents and exposure of equipment to potentially damaging solvents. Also, these steps complicate the assay and lengthen the time required to complete it. On the other hand, the aspiration/solubilization steps required for insoluble formazans can actually be an advantage if high background optical densities in the assay wells is as potential problem.

The new anticancer drug screening program of the National Cancer Institute requires a suitable assay system for use in large-scale screening. At the projected capacity of this program, perhaps as many as 10 million microculture wells per year have to be evaluated. Among the end-point assays being evaluated are the tetrazolium-based systems.



XTT, 1 2



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The feature of **1**, which first distinguishes it from the tetrazolium salts already in wide use, is that bioreduction of **1** produces a highly water soluble formazan **2** [11], thus allowing its use in assay protocols where a solubilization step can be eliminated. The presence of the two fully ionized sulfonate groups in **1** creates a net charge of −1. This is sufficient for water solubility. Bioreduction of **1** destroys the +1 charge of its tetrazolium nucleus, but this leaves a charge of −2 on the formazan **2**. It is this charge that renders **2** highly water soluble. There are, of course, additional properties required of a successful tetrazolium/formazan system: the tetrazolium salts must have very little color, but the formazan must have an intense color of an appropriate wavelength; the tetrazolium must be adequately bioreducible by a large number of cell lines, but extensive overreduction of the formazan must not occur; moreover, the tetrazolium salt and the formazan must both have very low toxicity to the cells used. As part of a continuing study of tetrazolium reagents, a pyridine-containing tetrazolium reagent reported to produce a water-soluble formazan [13] was synthesized and evaluated. When compared directly to **1** in four different cell lines of varying metabolic reductive potential under various conditions and scanned at wavelengths ranging from 450 to 630 nm, this pyridine compound was not useful for microculture tetrazolium growth/growth inhibition assays.

Chemistry.

Formazan **2** was prepared as previously outlined [3]. The key intermediate, the nitroaniline **5**, was prepared from 3-amino-4-methoxybenzenesulfonic acid **8** by a literature method [14]. The diazonium salt **3**, prepared from **5**, was treated at *p*H 4.9 to 5.5 with malonanilic acid **4**. Formazan **2** was isolated by filtration. A product suitable for the next step was obtained by a single crystallization of this product from ethanol/water. However, the analytically pure specimen of **2** was obtained by ascorbic acid reduction of the pure **1**.

Tetrazolium salt **1** was prepared by hypochlorite oxidation of **2**. The crude product was usually isolated by filtration in less than 30% yield. It is likely that the low yield from this oxidation step is related to the inability of some isomeric and tautomeric forms of **2** to cyclize [15]. Analytically pure material was obtained by a single crystallization from ethanol/water.

EXPERIMENTAL [16]

The physical properties were determined with the following instruments: melting point, Fisher-Johns apparatus (uncorrected); ir, Beckman "IR-33"; uv, Beckman "Acta CV"; ^{1}H nmr Brucker AM 500, (residual DMSO in DMSO-d_6, at 2.49 ppm was used as an internal standard). Elemental analyses were preformed by Galbraith Laboratories, Inc., Knoxville, TN; tlc, E. Merck silica gel 60 F-254 precoated glass plates were used to determine the purity of compounds. The spots on tlc were detected by uv (Spectroline Model ENF-26, 254 nm).

Malonanilic Acid (**4**).

A solution of aniline (280 g, 3.01 moles) and diethyl malonate (950.0 g, 5.930 moles) was heated to 156° as 150 ml of ethanol was distilled out. The solution was then cooled to 95°, and 10*N* sodium hydroxide (1000 ml) was added at such a rate that the newly formed ethanol (300 ml) was distilled off during 1 hour. The resulting mixture was diluted to 3 ℓ with water. The solid that separated was filtered off, and the filter cake was washed with water (1 ℓ). The filtrate was acidified with concentrated hydrochloric acid (500 ml), then stored for 4 hours at room temperature. The crystals that separated were collected on a filter, washed with water (2 x 100 ml), then dried *in vacuo* at 34° to give 118.5 g (22%) of product, mp 134-135°.

3-(*N*-*p*-Toluenesulfonamido)-4-methoxybenzenesulfonic Acid, Sodium Salt (**7**).

To a stirred solution of 3-amino-4-methoxybenzenesulfonic acid (**8**) (100 g, 0.492 mole) in pyridine (500 ml) was added *p*-toluenesulfonyl chloride (98.4 g, 0.516 mole) at room temperature in one portion. The temperature of the reaction mixture rose to 60°. The reaction mixture was heated at 55° for 8 hours, stored at room temperature for 16 hours,

then spin-evaporated *in vacuo* to a thick brown oil. To the oil was added water (150 ml) and 10% sodium hydroxide (500 ml). The resulting solution was extracted with ether (5 x 200 ml). The ether extracts were discarded, and the aqueous layer was concentrated *in vacuo* to a semi-solid (253 g). This material was used without further purification in the next step.

3-(*N*-*p*-Toluenesulfonamido)-4-methoxy-6-nitrobenzenesulfonic Acid, Sodium Salt (**6**).

To a stirred suspension of 3-(*N*-*p*-toluenesulfonamido)-4-methoxybenzenesulfonic acid sodium salt (**7**) (180 g) in dichloromethane (800 ml) at 0° was added sulfuric acid (17 ml) dropwise. To the resulting mixture was added the nitrating mixture (80 ml sulfuric acid: 25 ml nitric acid), dropwise, at 0-5° during 1 hour. The reaction mixture was stirred at 0-5° for 1 hour then heated at reflux for 90 minutes. The reaction mixture was cooled to 0-5°, and the supernatant dichloromethane solution was decanted. To the orange-red residue was added saturated aqueous sodium chloride (900 ml) at 0° with vigorous stirring. The solid was collected on a filter and washed with aqueous sodium chloride (3 x 100 ml). The solid was dissolved in acetone (1 ℓ), and the inorganic salts were filtered off and discarded. The filtrate was concentrated to a volume of 400 ml then cooled to 0-5°. The crystallized nitro derivative was collected on a filter, washed with acetone-dichloromethane (1:5) (3 x 50 ml), and then dried to give 84 g (43%) of the sodium salt.

3-Amino-4-methoxy-6-nitrobenzenesulfonic Acid (**5**).

A stirred suspension of 3-(*N*-*p*-toluenesulfonamido)-4-methoxy-6-nitrobenzenesulfonic acid, sodium salt (**6**) (50.0 g, 0.118 mole) in 20% v/v aqueous hydrochloric acid (1 ℓ) was heated at reflux for 3 hours. The resulting solution was clarified by filtration while hot, then cooled in an ice-bath for 2 hours. The precipitated solid was collected on a filter, washed with 20% v/v aqueous hydrochloric acid (1 x 50 ml), acetone (6 x 30 ml), then dried to constant weight *in vacuo* to give 18.7 g (64%) of purified product, mp >300°.

3-Diazo-4-methoxy-6-nitrobenzenesulfonic Acid (**3**).

A stirred suspension of 3-amino-4-methoxy-6-nitrobenzenesulfonic acid (**5**) (100 g, 0.403 mole) in water (700 ml) was neutralized by the portionwise addition of sodium hydroxide (16.4 g, 0.403 mole) in water (200 ml). The suspension was cooled to 5°, then a solution of sodium nitrite (27.8 g, 0.403 mole) in water (100 ml) was added. The resulting thick suspension was added, portionwise, to ice-cold aqueous hydrochloric acid (74 ml of concentrated hydrochloric acid: 600 g of chipped ice) during 15 minutes. The resulting finely divided suspension was stirred at 0-10° for 90 minutes, then used immediately in the next step.

3,3'-[3-(Phenylamino)carbonyl]-1,5-formazandiyl]bis(4-methoxy-6-nitro)-benzenesulfonic Acid, Disodium Salt (**2**).

A stirred solution of malonanilic acid (**4**) (36.1 g, 0.202 mole) in acetate buffer (1000 ml, *p*H 4.9) was cooled to 0°. The cold (3-5°) diazonium salt **3** suspension was added to the buffered solution in a thin stream during 30 minutes. During the addition, the internal temperature was maintained at 0-5°, and the *p*H was maintained at *p*H 4.9-5.3 by addition of 20% sodium hydroxide (*ca.* 115 ml). The resulting suspension was stirred at room temperature for 3 hours. During this time, the suspension became dark red and considerably thicker. The reaction mixture was heated to 35°, diluted with ethanol (4.0 ℓ), stored at 5° for 15 hours, then separated by filtration. The red solid was washed on the funnel with ethanol (500 ml), then recrystallized from ethanol-water (3.5:2.0 ℓ) to give 93.2 g (66%) of product suitable for further transformation, mp 296° dec; ir (potassium bromide): 450, 1640, 1600, 1530, 1450, 1350, 1250, 1055, 1020, 800 cm^{-1}; nmr (500 MHz) (DMSO-d_6): δ 14.88, 14.40, 12.21, 10.40, (s, heteroatom H), 8.36-7.16 (a complex 23 peak pattern in aromatic region); 4.17, 4.11, 4.09 (s, $-OCH_3$); uv (water): max 477 (log ϵ 4.037), 346 (4.098), 296 (4.232), 253 (4.263) nm.

Preparation of Analytically Pure **2** from **1** by Ascorbic Acid Reduction.

To a stirred solution of ascorbic acid (271 mg, 1.54 mmoles) and sodium bicarbonate (259 mg, 3.08 mmoles) in water (15 ml) was added analytically pure sodium 3,3'-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (1.0 g, 1.4 mmoles) (**1**). The resulting suspension was stirred at room temperature for 1.5 hours during which time a dark red solution was formed. The reaction mixture was diluted with ethanol (45 ml) to form a thick red suspension. The red solid was collected, then recrystallized from 55 ml of ethanol/-water (2:1) to give 400 mg of **2**; nmr (500 MHz) (DMSO-d_6): δ 14.88, 14.40, 12.21, 10.40 (s, heteroatom H), 8.36-7.16 (a complex 23 peak pattern in aromatic region), 4.17, 4.11, 4.09 (s, $-OCH_3$); uv (water): max 475 (log ϵ 4.377), 359 (4.084), 297 (4.219), 253 (4.255) nm.

Anal. Calcd. for $C_{22}H_{17}N_7Na_2O_{13}S_2$ + 5.0 H_2O: C, 33.55; H, 3.46; N, 12.45; Na, 5.84; S, 8.14. Found: C, 33.64; H, 3.39; N, 12.19; Na, 5.90; S, 8.03.

Sodium 3,3'-[1-[(Phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic Acid Hydrate (**1**).

To a stirred solution of benzenesulfonic acid, 3,3'-[3-[(phenylamino)carbonyl]-1,5-formazandiyl]bis(4-methoxy-6-nitro), sodium salt (**2**) (120.0 g, 0.146 mole) in water (900 ml) at 18° was added aqueous sodium hypochlorite (2.1%, 1200 ml) slowly during 2 hours with constant stirring. The stirring was continued for 30 minutes, then the *p*H was lowered to 4.0 by the addition of 0.5*N* hydrochloric acid (*ca.* 100 ml). The reaction mixture was cooled in an ice-bath for 1 hour. The precipitated yellow solid was collected on a filter, washed with ice-cold methanol (100 ml), then dried *in vacuo* to give 26.5 g (27%) of product. Additional reactions were carried out to give a total of 172 g of product. This material was recrystallized from ethanol-water (3.4 ℓ:1.7 ℓ) to give 146.6 g (85% recovery) of analytically pure product, mp 285° dec; ir (nujol) 3450, 3420, 1705, 1690, 1640, 1295, 1240, 1090, 1045, 1015, 805, 750, 710, 660 cm^{-1}; nmr (500 MHz) (DMSO-d_6): ppm 11.70 (s, 1H, -NH-), 8.54 (s, 2H, aromatic H *o*- to $-NO_2$), 7.93-7.91 (m, 4H, aromatic H *m*- to $-NO_2$ and aromatic H *o*- to -NHCO-), 7.53-7.50 (t, 2H, aromatic H *m*- to -NHCO), 7.31 (t-1H, aromatic H *p*- to -NHCO), 3.83 (s, 6H, $-OCH_3$); approximately 0.7% ethanol is observed in the spectrum; uv (water): max 285 nm (log 4.193); shoulder 221 nm (4.603); tlc: moves as one spot on silica gel (E. Merck 60 F-254 Glass Plates) when eluted with ethyl acetate 1-propanol water (3:2:1) giving Rf = 0.60 or with 1-butanol acetic acid water (4:1:1) giving Rf = 0.31 (some trailing).

Anal. Calcd. for $C_{22}H_{16}N_7NaO_{13}S_2$ + 1.5 H_2O: C, 37.72; H, 2.73; N, 14.00; Na, 3.28; S, 9.15. Found: C, 37.78; H, 2.76; N, 13.89; Na, 2.88; S, 9.20.

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[16] The unprecedented scale of the *in vitro* program conceived of and directed by M. B. led R. S. to focus on tetrazolium assay technology and to define the need for a water-soluble formazan. A general design stragety was developed by K. P. The initial small sample of formazan **2** was selected from the NCI repository by K. P. The authors thank M. G. for this sample. The first small-scale synthesis of **1** from **2** was achieved by authors D. B. and E. H. whose work was funded by NCI contract NO1-CM-27571. The authors thank Dr. Roger Haugwitz, Project Officer of that contract for his cooperation in this project. The large-scale synthesis was achieved by authors J. L. P., P. A. R., W. A. B. and M. N. S. whose work was funded by NCI contract NO1-CM-67926. The authors thank Dr. Karl Flora, the Project Officer for that contract, for his cooperation. The biological evaluation of XTT was performed by D. S., M. A. and A. M. whose work was supported by NCI contract NO1-CO-23910.