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5-(3-CARBOXYMETHOXYPHENYL)-2-(4,5-DIMETHYLTHIAZOLYL)-3-(4-SULFOPHENYL)TETRAZOLIUM, INNER SALT (MTS) AND RELATED ANALOGS OF 3-(4,5-DIMETHYLTHIAZOLYL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) REDUCING TO PURPLE WATER-SOLUBLE FORMAZANS AS CELL-VIABILITY INDICATORS

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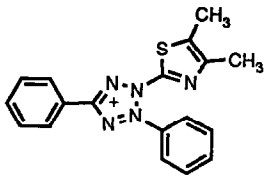
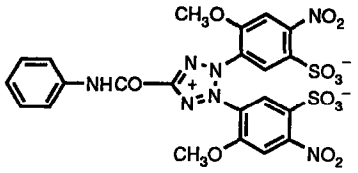
Abstract. Analogs of MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide, designed to yield water-soluble formazans upon reduction, have been synthesized and evaluated as cell-viability indicators.

Tetrazolium salts have been used for many years to distinguish living cells from dead ones. They are reduced to formazans by the cytochrome systems of viable cells, and the color developed is a direct measure of the viability of the culture. Of a large number of tetrazolium-formazan couples reported in the literature[1] about half a dozen have gained wide acceptance. Among these is the dimethylthiazolyl compound MTT (**1a**)[2]. It is readily reduced, often not requiring a redox intermediary such as phenazine methosulfate (PMS), and its formazan (**2a**) is deep blue (λ_{max} 565 nm). Formazans are exceedingly insoluble in water even at uv-vis spectroscopic concentrations, so that color evaluation has required microscopic examination of the cells in which the dye is deposited or, more recently,[2,3] solubilization protocols designed to bring the color into solution for spectrophotometric measurement. Water-soluble formazans offer the possibility of circumventing these post-incubation manipulations, and there has been interest for some time in developing tetrazolium compounds which would generate them. Most successful has been the sulfonated nitrophenyl compound XTT,[4] which now is used routinely in the screening of potential anti-AIDS drugs.[5] Variants of MTT would be expected to produce much more deeply colored water-soluble formazans, and in this and other respects might be useful with a greater variety of cell lines, particularly the wide spectrum of tumor and leukemia cells now capable of being grown in culture. We draw attention here to one compound in particular which exhibits significant promise, compound **1b**, for

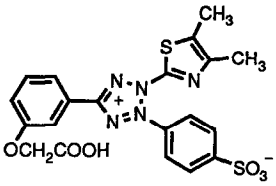
which the designation MTS is proposed.



MTT (1a)



XTT



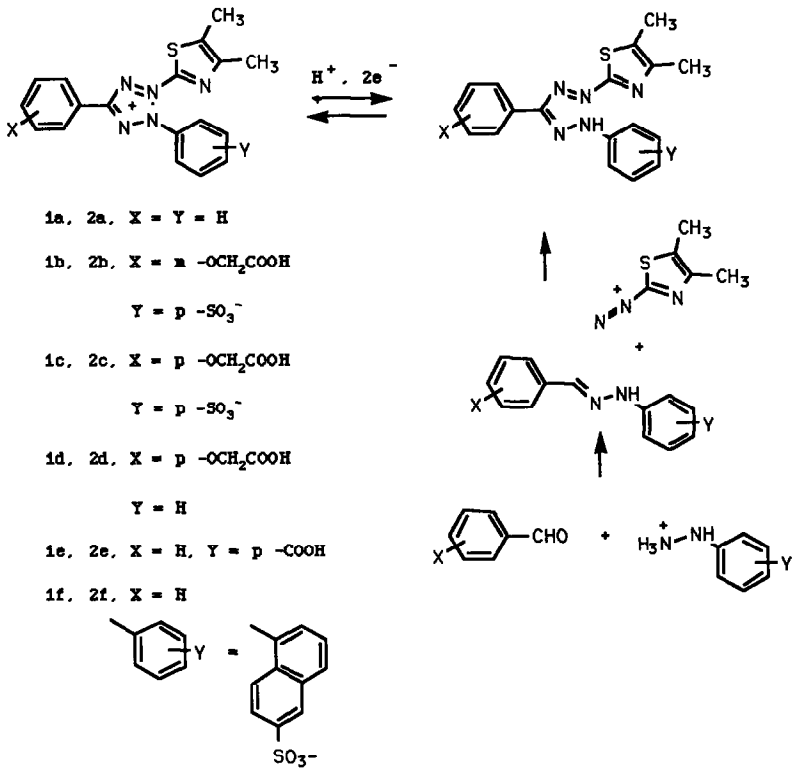
MTS (1b)

The route to the compounds of interest is shown in Scheme I.[6] Condensation of *m*-formylphenoxyacetic acid with *p*-sulfophenylhydrazine in aqueous pyridine gave *m*-formylphenoxyacetic acid *p*-sulfophenylhydrazone (sodium salt hemihydrate, from water, mp 285-288°, CHN, ^{1}H NMR) which, at -15°C with 4,5-dimethyl-2-thiazolediazonium chloride (from 2-amino-4,5-dimethylthiazole hydrochloride, sodium nitrite and 5 M hydrochloric acid at 0-5°C) gave the crude formazan **2b** (precipitated with brine as the disodium salt, 70% pure by uv-vis). Oxidation with bromine in aqueous acetonitrile effected rapid decolorization and precipitated tetrazolium betaine **1b** [yellow needles from acetonitrile-water, mp 260°C (decomp), λmax (water) 382.4nm ε 8,300, CHN]. An analytical sample of the hydrazine salt (hemihydrate) of formazan **2b** was prepared by reducing tetrazolium betaine **1b** with hydrazine [black-purple micro needles from 95% ethanol, mp 159°C (decomp), λmax (95% ethanol) 567.8 nm ε 19,800, λmax (water) 490.8 nm ε 27,500 sh 550-570 nm, CHN]. Similarly prepared were compounds **1c** [mp 274°C (decomp), λmax (water) 381.6 nm ε 7500, CHN]; **1d** [bromide mp 195°C, λmax (water) 378.4 nm ε 7500, CHN]; **1e** [bromide mp 222°C (decomp), λmax (95% ethanol) 377.6 nm ε 8100, CHN]; **1f** [monohydrate, dihydrate, phase change 240°C, decomp 360°C, λmax (water) 375 nm ε 11,000, CHNS]; **2c** [bis-$N_2H_5^+$ salt, black crystals from 95% ethanol mp 161-162°C (decomp), λmax (95% ethanol) 592.0 nm ε 17,100, λmax (water) 488.8 nm ε 29,900, CHN]; **2d** [black needles from toluene mp 206°C (decomp), λmax (95% ethanol) 579.2 nm ε 16,900, λmax (water) 492.0 nm ε 22,700, CHN]; **2e** [(black needles from xylene mp 218-219°C (decomp), λmax (95% ethanol) 568.4 nm, ε 21,000, λmax (pH 7 buffer) 497.6 nm, λmax (pH 10 buffer) 528 nm ε 21,500, CHN]; **2f**[not isolated; reduction of **1f** indicates λmax (95% ethanol) 591.2 nm ε 18,500, λmax (water) 505 nm]. Samples of MTT (**1a**) and its formazan (**2a**) were prepared by the same route.

A number of constraints, often mutually conflicting, affect the choice of functional groups which may be utilized to confer the needed water-solubility, spectroscopic characteristics and redox behavior. Thus, although formazan sulfonic acids such as **2f** are freely soluble, tetrazolium betaines such as **1f** are not. Salts, such as **1d**, of tetrazolium carboxylic acids are soluble in acidic water. At neutral pH, however, they also become sparingly soluble zwitterions; and their formazans are sparingly soluble even at pH 7.5. Two sulfonate groups, as in XTT and in other compounds claimed in the patent literature,[7] render both

the tetrazolium and the formazan freely soluble, of course, but it is common experience that such compounds are difficult to purify. Further complications are introduced by the instability of formazans to acids and of tetrazoliums to bases. The phenoxyacetate group, as in compounds **1b**, **2b**, **1c** and **2c** overcomes these problems nicely. It is more acidic (pKa ~ 3.5) than the ordinary carboxyl group, and so is completely protonated in dilute mineral acid but completely dissociated in pH 7 buffer. In consequence, oxidation of the crude formazan (**2b**, **2c**) sodium salts with bromine water precipitates, from the acidic reaction mixture, the sparingly soluble betaine carboxylic acids (**1b**, **1c**) which may be recrystallized from acidulated aqueous acetonitrile. The betaines store well and dissolve readily in pH 7 buffers and culture media as the dianion-monocation salts. Upon reduction they revert to the soluble formazan dianions. Analytically pure samples of the hydrazinium salts of the formazans (**2b**, **2c**) were prepared by reducing the pure tetrazolium compounds with aqueous hydrazine.

Scheme I



Compounds **1b-1f** have been evaluated in comparison with MTT using mouse leukemia L1210 cells, mouse Ehrlich tumor cells, human colon tumor HT-29 cells, and mouse 3T3-L1-Rosen fibroblasts. Details are reported elsewhere.[8] Compound **1b** was especially satisfactory, giving color yields at 490-560 nm comparable with those obtained with MTT in all cases without need for any post-incubation manipulation or solubilization. The other tetrazolium compounds gave much poorer results. The **para** isomer **1c** gave barely one third of the color yielded by **1b** and compounds **1d, 1d, 1e** and **1f** gave no useful color at all. These structure-function relationships are under investigation.

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6. New compounds 1b - 1f and 2b - 2e were authenticated by elemental analysis (CHNS), ^{1}H NMR and ^{13}C NMR.

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