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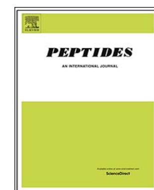

Research paper

Glutathione salts of O,O-diorganyl dithiophosphoric acids: Synthesis and study as redox modulating and antiproliferative compounds

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ABSTRACT

Reactions of glutathione (GSH) with O,O-diorganyl dithiophosphoric acids (DTPA) were studied to develop bioactive derivatives of GSH. Effective coupling reaction of GSH with DTPA was proposed to produce the ammonium dithiophosphates (GSH–DTPA) between the NH_2 group in γ-glutamyl residue of GSH and the SH group in DTPA. A series of the GSH–DTPA salts based on O-alkyl or O-monoterpenyl substituted DTPA were synthesized. Enhanced radical scavenging activity of the GSH–DTPA over GSH was established with the use of DPPH assay and improved fluorescent assay which utilizes Co/H_2O_2 Fenton-like reaction. Similarly to GSH, the dithiophosphates induced both pro- and antioxidant effects in vitro attributed to different cellular availability of the compounds. Whereas extracellularly applied GSH greatly stimulated proliferation of cancer cells (PC-3, vinblastine-resistant MCF-7 cells), the GSH–DTPA exhibited antiproliferative activity, which was pronounced for the O-menthyl and O-isopinocampheolyl substituted compounds **3d** and **3e** ($IC_{50} \geq 1$ μM). Our results show that the GSH–DTPA are promising redox modulating and antiproliferative compounds. The approach proposed can be extended to modification and improvement of bioactivity of various natural and synthetic peptides.

1. Introduction

L-γ-Glutamyl-L-cysteinyl-glycine (glutathione, GSH) is the prevailing antioxidant oligopeptide in mammals, which plays a crucial role in non-specific and enzyme-assisted defense of living cells from oxygen radicals, detoxification of xenobiotics, maintenance and regulation of the redox homeostasis in cells [1–4].

The association of GSH deficiency with a variety of human metabolic, degenerative, aging-related diseases [4] and viral infection [3] is well established. At the same time, elevated GSH level is often involved in tumor resistance and progression [5]. GSH, its analogues and metabolizing enzymes attract considerable interest in biomedical and pharmacological research. Most enzymes of the GSH metabolism, e.g. catalyzing biosynthesis of GSH (γ-glutamyl transferase), nucleophilic addition reactions of the thiol group (glutathione-*S*-transferase), methylglyoxal detoxification (glyoxalase) and redox reactions (glutathione reductase, glutathione peroxidase) are established pharmacotherapeutic targets [1,6].

A number of synthetic approaches to developing enzyme effectors based on the tripeptide GSH have been proposed to date. Main strategies for generation of the bioactive analogues and derivatives of GSH, summarized by Lucente et al. [1], include replacement of one or more amino acids in the tripeptide backbone with artificial analogues (*D*-, N-methyl-, α-methyl-glutamic acid, α-methyl-L-cysteine, β-alanine) as well as modification of the SH group of cysteine in order to produce both reversible and irreversible inhibitors of GSH-metabolizing enzymes (see [1] and references within). Some other approaches in GSH chemistry proposed are aimed at the GSH derivatives with increased biological stability, e.g. resistance to blood γ-glutamyl transpeptidase, by means of esterification of the carboxyl functions [6] and cyclization of the GSH molecule [1].

Burg and Mulder summarized the state of the art in developing derivatives and analogues of GSH with antiproliferative activity and studying their capacity to overcome cancer drug resistance associated with GSH-dependent enzymes [6]. These enzymes are glutathione *S*-transferases catalyzing GSH conjugation to harmful electrophilic compounds, including carcinogens and anticancer drugs, DNA-dependent protein kinase (PI 3-kinase family) involved in repairing double-strand breaks in DNA as well as glyoxalase system (glyoxalases I and II) participating in elimination of cytotoxic α-oxoaldehydes [6]. Some examples of effective inhibitors of these enzymes include *S*-alkyl derivatives of γ-glutamyl-2-aminoadipic acid analogue, GSH derivatives

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modified with phenyl and phosphonic groups, *S*-*p*-bromobenzyl-glutathione (see [6] and references within).

Considerable efforts are being made to utilize therapeutic potential of GSH and its precursors as intracellular antioxidants and regulators of the redox state in mammalian cells. Involvement of the oxidative stress and concomitant GSH depletion was shown for many disorders including those affecting skin, leaver, pulmonary and ocular tissues as well as preeclampsia, myocardial ischemia, neurodegenerative diseases, and decreased immune function [4,7]. Restoring balance between the reduced form of glutathione (GSH) and the oxidized one (GSSG) by using precursor compounds helps to diminish the oxidative injury and its pathological consequences [2,4,7].

Since a relatively low pharmacotherapeutic potential of GSH per se upon both oral and parenteral administration [4], prodrugs and derivatives of GSH with improved pharmacokinetic properties are being discovered. These compounds are particularly targeted at antiviral therapy against retroviruses, including HIV, influenza, rhinovirus, herpes simplex virus [3]. *N*-acetyl-cysteine (NAC), β-mercaptoethylamine derivative of NAC, GSH monoethylester, *S*-acetylglutathione, and *N*-butanoyl GSH were shown to act as prodrugs which increase intracellular level of GSH and other thiols and exert antiviral effects (see [3] and references within).

S-trityl-L-cysteine derivatives [8] and alkyl chain modified GSH derivatives coupled through *N*-terminal NH_2 group [9] with increased inhibitory activity towards hepatitis C and herpes simplex viruses, respectively, were synthesized. Antiviral activity of the GSH derivatives could result from both direct inhibition of the viral replication at different stages and immunomodulatory activity of the compounds [3,10]. *S*-acyl glutathione derivatives with fatty acids, such as *S*-lauroylglutathione and *S*-palmitoleoylglutathione, were recently developed as potential therapeutics in neurodegenerative diseases. These compounds have increased cellular availability, protect SH-SY5Y cells and cholinergic neurons against amyloid-induced oxidative damage and apoptosis via regeneration of intracellular pool of GSH [11,12].

In spite of a number of bioactive derivatives of GSH reported there is, however, the lack of the approved drugs which encourages development of alternative approaches to modification of GSH. To date, different strategies to modify peptide based drugs have been elaborated [13]. Organophosphorus compounds are promising modifiers for natural and synthetic peptides, which allow for improvement of their physicochemical and biological properties. Among them, dithiophosphoric acids (DTPA) are insufficiently explored compounds with previously established insecticide [14], antimicrobial activities [15–17], and which are relatively low-toxic for warm-blooded animals in contrast to phosphoric acid esters [14].

Different DTPA derivatives with natural alcohols, phenols and monoterpenes were earlier synthesized [15–18]. These DTPA, being a huge lipophilic anion, can form ammonium dithiophosphates with hydrophilic amino acids as a result of quaternization of the most basic nitrogen atom by the acidic SH group of DTPA. It was assumed that the reaction of O,O-diorganyl DTPA with GSH will improve properties of the tripeptide and generate new bioactive compounds. We provide proof-of-concept of this chemical approach and study effects of the dithiophosphoryl groups on antioxidant and antiproliferative properties of the GSH derivatives.

2. Material and methods

2.1. Materials

Tetramethylrhodamine, ethyl ester, 2′,7′-dichlorofluorescin diacetate, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. BODIPY 581/591 C11 lipid peroxidation sensor was obtained from Thermo Fisher Scientific. Reduced glutathione (purity 98%) and oxidized glutathione (purity 95%) were purchased from Acros Organics.

(*S*)-(–)-menthol (purity 99.5%) was purchased from Acros Organics. (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (purity 98%) was purchased from Sigma-Aldrich.

Materials for cell culturing were obtained from PAA Laboratories. Milli-Q grade water (Milli-Q® Advantage A10, Merck Millipore) was used to prepare buffers and solutions.

2.2. Synthesis of O,O-diorganyl dithiophosphoric acids

O,O-Dialkyl dithiophosphoric acids **2a** (δ_P 85.4 ppm), **2b** (δ_P 83 ppm) and **2c** (δ_P 86.4 ppm) were synthesized as described previously [19].

O,O-Bis[(–)-(1R,2S,5R)-2-isopropyl-5-methylcyclohex-1-yl] dithiophosphoric acid (**2d**) was synthesized by the reaction of tetraphosphorus decasulfide with (*S*)-(–)-menthol ($[\alpha]_D^{20}$–20.8°, c 1.035, C_6H_6), mp 61–63 °C, δ_P 81.3 ppm, C_6H_6) as reported recently [20].

O,O-Bis[(+)-(1*S*,2*S*,3*S*,5*R*)-trimethylbicyclo[3.1.1]hept-3-yl dithiophosphoric acid (**2e**) was synthesized as follows. Tetraphosphorus decasulfide (0.9 g, 8.3 mmol) was added portionwise under dry argon with stirring at 20 °C to the solution of (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (1.7 g, 11 mmol) in 20 mL anhydrous benzene, and stirring was continued for 2 h at 50 °C. The mixture was stored at 20 °C overnight and filtered. The filtrate was evaporated at reduced pressure (0.5 mm Hg) at 40 °C for 1 h and then in vacuum (0.02 mm Hg) for 1 h to give **2e** (1.8 g, 77%). Acid **2e** was purified by use of a column chromatography (silica gel 0.060–0.200 µm, eluent − benzene). R_f 0.45 (hexane). n_D^{20} 1.5230. $[\alpha]_D^{22}$ +35.0° (c 1.0, C_6H_6). FTIR (cm^{-1}, liquid film): ν_{max} 2987_{st}, 2911_{st} (CH_3 as, s; CH_2 as, s), 2583_w (S–H, free), 2403_w (S–H $_{related}$), 1471_{st}, 1452_{st} δ (CH_3 as); 1385_m, 1368_m δ [$(CH_3)_2C$ gem. s], 1089_m [(P)O–C], 973_{st} (OC–C, C–C), 772_{st} (PO_2 as, s), 676_{st} (P = S), 521_m (P–S). ^1H NMR ($CDCl_3$) ppm: δ = 0.96 and 1.23 [two s, 12H, $(C^{9',10'}H_3)_2C$], 1.21 (d, 6H, $C^{8'}H_3$, $^3J_{HH}$ = 7.5 Hz), 1.97 (m, 4H, $C^{7'}H_2$), 2.24 (m, 2H, $C^{2'}H$), 2.38 (m, 4H, $C^{6'}H_2$), 2.62 (m, 2H, $C^{3'}H$), 4.96 (m, 2H, P-O$C^{1'}H$). ^{31}P-{^1H} NMR (benzene) ppm: δ = 85.1. Anal. Calcd. for $C_{20}H_{35}O_2PS_2$: C, 59.67; H, 8.76; P, 7.69; S, 15.93. Found: C, 59.98; H, 8.33; P, 7.58; S, 15.73%.

2.3. Synthesis of glutathione salts of dithiophosphoric acids (3a–3e)

2.3.1. General procedure

Dithiophosphoric acid **2** (0.81 mmol) was added drope wise under dry argon with stirring at 20 °C to the solution of glutathione **1** (0.81 mmol) in 15 mL anhydrous ethanol, and stirring was continued for 1.5 h at 50 °C. The mixture was filtered and the filtrate was evaporated at reduced pressure (0.5 mm Hg) at 40 °C for 1 h and then in vacuum (0.02 mm Hg) to give **3**. Volatiles were removed under reduced pressure.

The synthesis of **3a–3e** is detailed in the Supplementary data.

2.3.2. Structure characterization

Melting points were determined on an electrothermal (variable heater) melting point apparatus and are uncorrected. The optical rotations recorded on a Perkin-Elmer 341 polarimeter (Norwalk, CT, USA) with a pathlength 55.2 mm using the λ = 589 nm of *D*-line of sodium. FTIR spectra were obtained in film with a Bruker Tensor 27 infrared spectrophotometer (Bruker BioSpin AG, Fällanden, Switzerland) (400–4000 cm^{-1}) and expressed in cm^{-1}, δ = the deformation vibration, st = strong, w = weak, m = medium vibrations, s – symmetric and as − asymmetric vibrations; gem – geminal. The ^{31}P NMR spectra were taken on a Bruker Avance-400 (161.9 MHz) instruments (Bruker BioSpin AG, Fällanden, Switzerland) in ethanol with 85% H_3PO_4 as an external reference. The ^1H (400 MHz), ^{13}C and ^{13}C-{^1H} (100.6 MHz) spectra were run at ambient temperature on a Bruker Avance (III) 400 instruments (Bruker BioSpin AG, Fallanden, Switzerland) in acetone-d_6. Chemical shifts (δ are given in parts per million (ppm) relative to

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