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Synthesis of two fluorescent GTPγS molecules and their biological relevance

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Abstract

Fluorescent GTP analogues are utilized for an assortment of nucleic acid and protein characterization studies. Non-hydrolysable analogues such as GTPγS offer the advantage of keeping proteins in a GTP-bound conformation due to their resistance to hydrolysis into GDP. Two novel fluorescent GTPγS molecules were developed by linking fluorescein and tetramethylrhodamine to the γ-thiophosphate of GTPγS. Chemical and biological analysis of these two compounds revealed their successful synthesis and ability to bind to the nucleotide-binding site of tubulin. These two new fluorescent non-hydrolysable nucleotides offer new possibilities for biophysical and biochemical characterization of GTP-binding proteins.

Keywords

Nucleoside and nucleotide; analogs; fluorescent; nucleotide analogs; FRET;; NMR and tubulin; polymerization

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Introduction

Nucleotides are one of the fundamental biological building blocks in living organisms and are used to assemble deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Ribonucleotides, particularly adenine and guanine nucleotides, have important roles in cell metabolism and signaling in addition to their function in RNA assembly. Hydrolysis of nucleotide triphosphates can be used to drive thermodynamically unfavorable reactions, as seen with kinases and ATP, or may induce conformational change of the protein to open up or block binding regions, as seen with G-proteins in signal transduction or with tubulin in polymerization when binding GTP or GDP [1].

Nucleoside and nucleotide analogs have uses therapeutically as cancer and antiviral treatments [2] and have also been used extensively in studies of nucleic acids and proteins to study biochemical interactions and mechanisms. The phosphate region of the nucleotide can be modified to render the GTP molecule resistant to hydrolysis, and these are often termed “non-hydrolysable” analogues. This type of GTP analog is useful for studies involving GTP-binding proteins in which the activity or structure of the protein in its GTP-bound conformation is preferred over its GDP-bound conformation. GTPγS is one such analog that is commercially available and is used in a variety of G-protein studies for its resistance to hydrolysis by GTPases [3].

Particularly useful are analogs labeled with a fluorophore. Fluorescent nucleotide analogs offer an alternative method to using radiolabeled nucleotides to study protein activity, removing the need to dispose of radioactive waste [4]. Common uses for fluorescent nucleotide analogs for the study of nucleic acids include interaction of enzymes and other proteins with nucleic acids, detection of oligonucleotide hybridization, and examining DNA structural conformations through energy transfer experiments [5]. For proteins, fluorescent nucleotides have been used to obtain kinetic information of nucleotide binding and dissociation, to study competition of ligands with the nucleotide-binding site of and for structural characterization [6].

Nucleotides linked to a variety of fluorophores can be purchased or synthesized, which allows for flexibility during assay development. This is particularly useful when designing Fluorescence Resonance Energy Transfer (FRET) experiments. FRET is the non-radiative transfer of energy from a donor fluorophore to an acceptor fluorophore. After excitation of the donor, energy may be transferred to the acceptor if it is within close enough proximity. For standard FRET applications, the distance between fluorophores where energy transfer can occur is usually within the range of 1–20 nm [7].

Here, we describe the synthesis, characterization, and biological application of two fluorescent GTPγS molecules, FGTP and TGTP. Fluorescein and tetramethylrhodamine were chosen as the fluorophores for FGTP and TGTP, respectively, for use in energy transfer studies since they are known FRET pairs with an R_0 of 5.5 nm [8]. The fluorophores are connected to the GTPγS molecule at the γ-phosphate via a thioether linkage. This synthesis has been shown with other thiolreactive fluorophores [9–11], and we wanted to observe if this could be recapitulated with fluorescein and tetramethylrhodamine. These functional

nucleotides have the possibility to be used in a variety of fluorescence-based applications from structural characterization to examining molecular interactions of GTP-binding proteins *in vitro*.

Methods

FGTP and TGTP synthesis and purification

Two bottles of GTPγS tetralithium salt (10 mg, Sigma Aldrich) were each dissolved in 1 mL H_2O, and buffered to pH 8.0 with $NaHCO_3$. 4.5 mg of 5-Iodoacetamidofluorescein (5-IAF) and 5 mg Tetramethylrhodamine-5-iodoacetamide (5-TMRIA) (Setareh Biotech) were each dissolved in 200 μL DMF, and the volume was brought to 1 mL with H_2O. The 1 mL solutions of 5-IAF and 5-TMRIA were each mixed with 1 mL of buffered GTPγS in H_2O. Reactions were vortexed briefly and put on a nutator overnight at room temperature. Reactions were assessed for completeness by reverse phase thin-layer chromatography as described below. The products FGTP (from the 5-IAF reaction) and TGTP (from the 5-TMRIA reaction) were purified on a C18 prep column (XTerra Prep MS C18 OBD, 5 μm, 19 × 150 mm). Product peak fractions were pooled and lyophilized and analyzed by NanoDrop 1000 UV-Vis, with peaks corresponding at λ = 254 nm and 470 nm for FGTP, and λ = 254 and 550 nm for TGTP.

Thin-layer chromatography

A total of 2 μL of 5-IAF, 5-TMRIA, FGTP, and TGTP in solution were spotted on a reverse phase thin-layer chromatography glass plate (Analtech RPS UNIPLATE, 250 μm, 5 × 20 cm). The 5-IAF and 5-TMRIA were co-spotted with 2 μL of GTPγS in solution. The 5-IAF and FGTP-spotted plates were placed in a mixture of water/methanol/acetic acid (67:28:5). 5-TMRIA and TGTP-spotted plates were placed in a mixture of water/methanol (40:60). After plates were developed and allowed to dry, they were visualized under a 365 nm UV lamp. FGTP migrated with an R_f of 0.89while 5-IAF migrated with an R_f of 0.26. Similarly, TGTP migrated with an R_f of 0.89 while 5-TMRIA migrated with an R_f of 0.29. These results suggest that the fluorophores were successfully attached to the GTPγS molecule, observed by the increased hydrophilic character of the new compounds compared to dye alone which allowed them to travel farther up the hydrophobic surface of the plate.

Analytical HPLC

The 5 mM solutions of GTPγS, 5-IAF, and 5-TMRIA were prepared, dissolving GTPγS in H_2O and then buffering to pH 8.0 with $NaHCO_3$, and dissolving 5-IAF and 5-TMRIA in DMF. Next, 3:1 molar ratios of GTPγS:fluorescent dye (5-IAF or 5-TMRIA) were mixed in 0.5 mL tubes, protected from light, and mixed overnight at room temperature on a nutator. The next day, samples were run on an analytical C18 reverse phase column (Phenomenex Luna® 5 μm, C18(2) 100 Å, LC Column 250 × 4.6 mm), running a gradient of 20 minutes H_2O, 5 minutes from 0% to 100% acetonitrile, 2 minutes from 0% to 100% H_2O, and 5 minutes at 100% H_2O at a flow rate of 1 mL/min. Absorbance was measured on a diode array detector at 254 nm.

Spectral analysis

Absorbance of FGTP and TGTP was measured on a NanoDrop 1000 Spectrophotometer using the “UV/Vis Absorbance” module. FGTP emission was measured on a NanoDrop 3300 Fluorospectrometer using Blue LED. TGTP emission was recorded using a Spectramax M2 microplate reader using an excitation wavelength of 540 nm. All spectra data was then normalized on a scale of 0 to 1 for comparison. Optimal excitation and emission values for FGTP and TGTP were determined using a Spectramax M2 microplate reader by performing excitation and emission scans for each compound.

Mass spectrometry

Electrospray Ionization Mass Spectrometry (ESI-MS) on FGTP and TGTP was performed by TriLink Biotechnologies, Inc. A 10 mM tertbutyl amine in 70% acetonitrile was run at a flow rate of 100 μL/min, with voltage at 4 kV and capillary temperature at 200°C.

Nuclear magnetic resonance (NMR) spectroscopy

For NMR analysis, 550 μL of 2 mM solutions of FGTP and TGTP were prepared by dissolving lyophilized FGTP and TGTP in PBS (comprised of 90% H_2O and 10% D_2O) with 2 mM TMSp (3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid). Solutions were placed into 5 mm NMR tubes. All NMR experiments were performed using a Bruker Avance-III 600 MHz NMR spectrometer equipped with a 5 mm CPTCI H/C/N/D Z-gradient cryogenic probe, with the temperature regulated at 298 K. Proton spectra were collected using the 1D NOESY water suppression method (noesypr1d Bruker pulse program) using 100 ms NOESY mixing time, a 12 ppm spectral width, 36k acquired points (2.5 second acquisition time), a 2.5 second relaxation delay, 128 scan averages and 8 dummy scans. 2DCOSY spectra were collected using the cosygpprqf Bruker pulse program, solvent presaturation during a 2 second relaxation delay, 4 scans and 16 dummy scans, 12 ppm spectral width, 4096 acquired points in F2 and 256 points in F1, and a 2 second recycle delay during which low-powered solvent presaturation was applied on the water resonance. All proton chemical shifts are referenced internally to TMSp at 0.0 ppm.

Displacement of [8-^{14}C]GDP from the E-site of tubulin

Electrophoretically homogeneous bovine brain tubulin was prepared as described previously [12], including gel filtration chromatography to remove unbound nucleotide [13]. The tubulin with [8-^{14}C]GDP bound in the E-site was prepared by doing an additional two cycles of assembly with 1.0 mM [8-^{14}C]GTP, followed by gel filtration chromatography [14]. See Lin et al. [15] for the preparation of GTPγS. The GDPβS was purchased from Boehringer-Mannheim. GTP was repurified by triethylammonium bicarbonate gradient elution from DEAE-cellulose and was at least 99% pure. Ansamitocin P-3 was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute.

Measurement of tubulin-bound [8-^{14}C]GDP was measured by centrifugal gel filtration, essentially as described previously [16]. Reaction mixtures (0.22 mL) contained 1.5 mg/mL (15 μM) tubulin with [8-^{14}C]GDP in the E-site, 0.1 M 4-morpholineethanesulfonate (taken from a 1 M solution adjusted to pH 6.6 with NaOH), 0.5 mM $MgCl_2$, and nucleotide as indicated. After 15 minutes on ice, 20 μM ansamitocin P-3 was added to each reaction

mixture, and 0.1 mL of the reaction mixture was applied to duplicate syringe-columns that contained Sephadex G-50 (superfine) swollen in 0.1 M 4-morpholineethanesulfonate (pH 6.6), 0.5 mM $MgCl_2$, and 10 μM ansamitocin P-3. The syringe-columns were centrifuged at 2,000 rpm for 4 minutes at 4°C (Allegra 6KR centrifuge, GH-3.8A rotor, Beckman-Coulter Instruments). Aliquots of the filtrates were counted in a scintillation counter and examined by Lowry reaction for their protein content. These data were used to determine the amount of [8-^{14}C]GDP bound to the tubulin. In control reaction mixtures there was an average of 0.77 pmol of [8-^{14}C]GDP per pmol of tubulin. The ansamitocin P-3 was used to minimize the amount of [8-^{14}C]GDP lost from the tubulin during centrifugation [17].

Tubulin polymerization assay

Each 50 μL reaction mixture contained 1.0 mg/mL (10 μM tubulin), 0.8 M monosodium glutamate (taken from 2.0 M solution adjusted to pH 6.6 with HCl), 0.5 mM $MgCl_2$, and nucleotide as indicated. Reaction mixtures were incubated at 30°C for 30 minutes and centrifuged at 30,000 rpm for 10 minutes at 30°C (Optima TLX ultracentrifuge, TLA 55 rotor, Beckman-Coulter Instruments). The supernatants were removed by aspiration, and the pellets were dissolved in 50 μL of 8 M urea. The protein content of the pellets was determined by the Lowry assay. Data were corrected for the amount of protein recovered in pellets from reaction mixtures without nucleotide (average, 2.0 μg). Glutamate-induced assembly [18] was measured to avoid the confounding effect of microtubule-associated proteins [14] on the total amount of tubulin assembled.

Results

The thiol group on the phosphate of GTPγS can react with dyes functionalized with a thiol-reactive group such as iodoacetamide. For the synthesis of FGTP and TGTP (structures in Fig. 1), fluorescein and tetramethylrhodamine functionalized with an iodoacetamide group (5-IAF and 5-TMRIA, respectively) were used as the reactive dyes. After mixing each respective dye and GTPγS, the condensation products were assessed by TLC and purified by HPLC once the appropriate reaction conditions were determined.

In addition to TLC, the successful synthesis of the new compounds and their ability to be purified separate from the starting compounds was examined using an analytical reverse-phase C18 HPLC column. First, starting materials were run to examine their elution profiles (data not shown). GTPγS eluted easily and quickly from the column with H_2O, while 5-IAF and 5-TMRIA eluted from the column with acetonitrile. When running the reaction mixtures, the new compounds FGTP and TGTP eluted after GTPγS and before 5-IAF or 5-TMRIA, which was expected (Figs. 2A and B). The reactions depicted were composed of GTPγS and 5-IAF or 5-TMRIA at a 3:1 ratio (GTPγS:fluorophore). The general height of the peaks of the reaction mixtures also suggests that most of the free fluorophore reacted with GTPγS to form a new compound when GTPγS was used in excess of the fluorophore.

UV-Vis analysis of FGTP and TGTP revealed that both the nucleotide and the fluorophore absorbance could be detected at approximately 254 nm for the nucleotide, and at 470 nm and 550 nm for FGTP and TGTP respectively, suggesting the fluorophore was successfully conjugated to the nucleotide (Fig. 3A). The excitation and emission profiles of FGTP and

TGTP agree with those for fluorescein and tetramethylrhodamine (Fig 3B). Optimal excitation and emission wavelengths for FGTP and TGTP were determined to be 487 nm/515 nm for FGTP and 540 nm/576 nm for TGTP. Also as expected, the absorbance spectrum of fluorescein from FGTP overlaps with the emission spectrum of tetramethylrhodamine from TGTP, demonstrating their use as FRET pairs (Fig. 3C).

Electrospray Ionization Mass Spectrometry revealed that the newly created compounds FGTP and TGTP have molecular weights of 926.8 Da and 980.4 Da, respectively. This closely matches the theoretical molecular weights of 926.59 Da for FGTP and 980.72 Da for TGTP (Figs. 4A and B).

^{1}H NMR was performed on both FGTP and TGTP to confirm the placement of the fluorophore and the structure of the nucleotide. The analysis revealed that both of the proposed chemical structures of FGTP and TGTP were correct with the fluorophores being positioned correctly at the γ-phosphate of GTPγS. ^{1}H NMR (D_2O) peaks for FGTP were: δ 8.04 (d, 1H, 53, CH), 8.03 (s, 1H, 10, CH), 7.63 (dd, 1H, 51, CH), 7.24-7.22 (m, 1H, 36, CH), 7.13 (d, 1H, 43, CH), 7.02 (d, 1H, 50, CH), 6.68 (s, 1H, 33, 46, CH), 6.68-6.66 (m, 2H, 35, CH), 6.62 (dd, 1H, 44, CH), 5.71 (d, 1H, 12, CH), 4.43 (t, 1H, 16, CH), 4.39 (dd, 1H, 15, CH), 3.82 (d, 2H, 61, CH_2). ^{1}H NMR (D_2O) peaks for FGTP were: δ 8.14 (d, 1H, 51, CH), 8.05 (s, 1H, 10, CH), 7.84 (dd, 1H, 49, CH), 7.43 (d, 1H, 48, CH), 7.15 (d, 1H, 43, CH), 6.80 (dd, 1H, 36, CH), 6.73 (dd, 1H, 44, CH), 6.35 (s, 1H, 34, CH), 6.28 (s, 1H, 46, CH), 5.72 (d, 1H, 12, CH), 3.87 (d, 2H, 59, CH_2), 3.11(s, 7H, 64, 65, CH_3), 3.09 (s, 6H, 60, 61, CH_3) (Figs. 5A and B).

FGTP and TGTP can bind to the nucleotide-binding pocket of GTP-binding proteins such as tubulin. Tubulin functions as a heterodimer, composed of α- and a β-tubulin subunits. Typically, nucleotide exchange occurs in the β-subunit. Thus, this nucleotide-binding site is often termed the “exchangeable” site, while the nucleotide-binding site in the α-subunit is termed the “non-exchangeable” site. Both subunits bind one molecule of GTP each; however, the β-subunit has the ability to hydrolyze GTP to GDP, and subsequently exchange the GDP for a new GTP molecule. This hydrolysis and exchange of the nucleotide is used to regulate the assembly and disassembly of microtubules [19].

TGTP and FGTP were examined for possible interactions with tubulin in two assays. In the first, we examined relative affinity of TGTP and FGTP for the exchangeable guanine nucleotide site (E-site) of tubulin by their ability to displace [8-^{14}C]GDP from the E-site in comparison with GTP, GTPγS, and GDPβS (Fig. 6A). Since affinity for the E site does not correlate with ability to induce tubulin assembly [14], we evaluated TGTP and FGTP for their ability to support tubulin assembly in comparison with GTP and GTPγS (Fig. 6B). Both analogues were able to displace GTP, but with lower affinity compared to the native nucleotide triphosphate. Consistent with an earlier study in which polyphosphate-modified analogues of GTP were demonstrated to have significantly reduced ability to displace radiolabeled GDP from tubulin [14], this apparent reduced affinity was also observed with TGTP and FGTP, as well as with GDPβS (Fig. 6A). The activity of GTPγS, however, was essentially identical to that of GTP.

It has been shown that analogs of GTP have varying binding affinities to tubulin and may either promote or inhibit tubulin polymerization [14, 16, 20–22]. GTPγS in particular is known to inhibit tubulin polymerization but can still bind to the nucleotide binding site of tubulin [16]. We examined whether the fluorescent conjugates of GTPγS developed in this study exhibited similar characteristics as non-conjugated GTPγS in regards to tubulin binding and ability to induce polymerization. Initially, we examined the ability of TGTP and FGTP to induce tubulin assembly in a standard turbidimetry assay at 350 nm. We found, however, that high concentrations of both compounds caused very erratic baselines in the assay. Nonetheless, it seemed clear that neither compound was able to support tubulin assembly at concentrations as high as 1.0 mM. We therefore modified a centrifugal assay that we have successfully used with taxoid and epothilone analogues [15]. We had previously found that measuring residual tubulin in the supernatant, using the Lowry assay, allowed us to readily distinguish between less active and more active drug molecules in an assay without GTP in the reaction mixture, where there was no assembly in control reaction mixtures. However, we found that both TGTP and FGTP interfered with the Lowry reaction, but we found that this problem was minimal if protein content of the pellet was measured. A summary of our findings is presented in Figure 6B. Increasing concentrations of GTP, up to 1.0 mM, led to an increasingly large protein pellet (about 60% of the tubulin in the reaction mixture was polymerized at the highest GTP concentration used). No significant assembly was observed with FGTP, TGTP, and GTPγS at concentrations as high as 1.0 mM. The inability of GTPγS to support assembly was in agreement with earlier studies that demonstrated that it was an inhibitor of tubulin assembly [16].

Discussion

FGTP and TGTP are two new fluorescently labeled GTPγS molecules that display the spectral characteristics of fluorescein and tetramethylrhodamine and maintain the ability to function as a nucleotide, as demonstrated by its ability to bind to tubulin. By using GTPγS for the production of FGTP and TGTP, these two fluorescent nucleotides should also be resistant to hydrolysis, though this has yet to be tested thoroughly. Such a characteristic would give these new compounds increased stability for protein studies that require the use of GTP over GDP. This would also give FGTP and TGTP an advantage over other guanine analogs that may be more susceptible to phosphate hydrolysis.

Fluorescent labels are commonly added onto the ribose moiety of guanine nucleotides, whereas FGTP and TGTP have the fluorophore attached to the γ-phosphate. In the case of tubulin, FGTP and TGTP both associate with the nucleotide-binding site, but the affinity of these analogs for tubulin is lower than native GTP, GTPγS, and some other analogs. The location of the fluorophore undoubtedly affects how the nucleotide binds to proteins, though whether it impedes binding or not needs to be determined empirically for each protein.

FGTP and TGTP were able to displace [8-^{14}C]GDP from the exchangeable site, suggesting that the fluorescent GTP analogs can fit into the nucleotide binding pocket of tubulin. FGTP and TGTP have a reduced affinity for the binding pocket as compared to native GTP and GTPγS, but have comparable affinities to other GTP or GDP analogs such as GTPβS and GDPβS. These findings show that FGTP and TGTP have the ability to be bound by GTP-

binding proteins, making them useful for fluorescence based assays for this category of proteins.

Conclusion

The work presented in this study details the synthesis of two fluorescent, hydrolysis-resistant guanosine triphosphate analogs for the study of GTP-binding proteins. Fluorescein and tetramethylrhodamine were attached to GTPγS at the γ-phosphate via a thioether linkage, which was confirmed through thin-layer chromatography, high-performance liquid chromatography, and nuclear magnetic resonance analyses. [8-^{14}C]GDP displacement studies also confirmed that the fluorescent nucleotides can associate with the GTP-binding protein tubulin. The simple method required for synthesis of FGTP and TGTP means that this protocol can be extended to other fluorophores containing iodoacetamide or other thiol-reactive groups to rapidly synthesize new fluorescently-tagged nucleotides.

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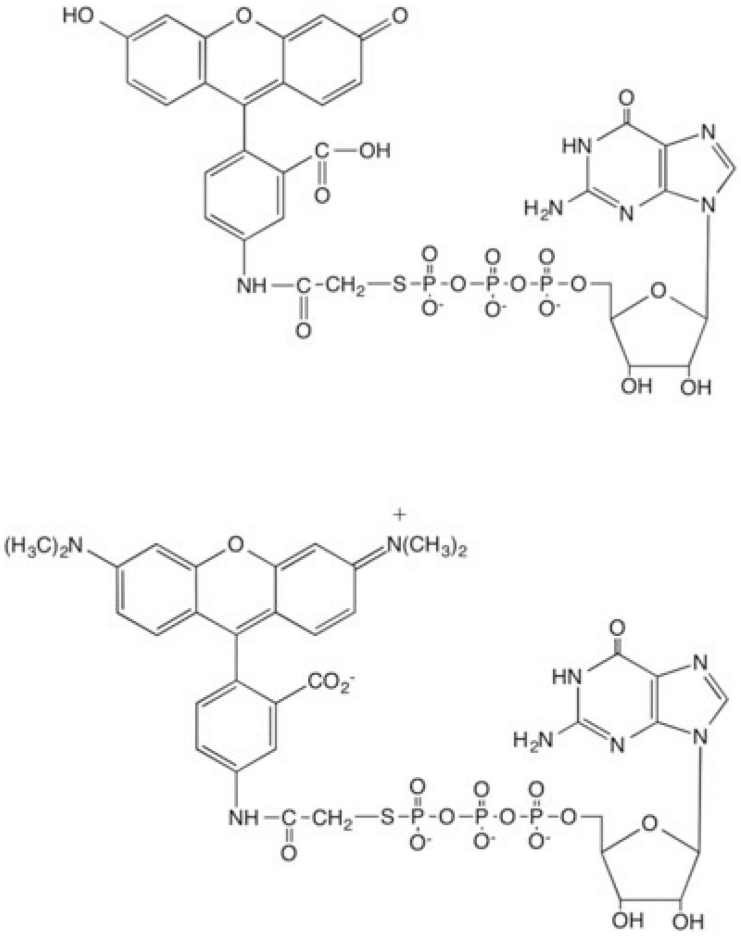


Figure 1.

Structures of FGTP and TGTP. GTPγS was combined with thiol-reactive dyes to produce two fluroescently labeled nucleotide analogs.

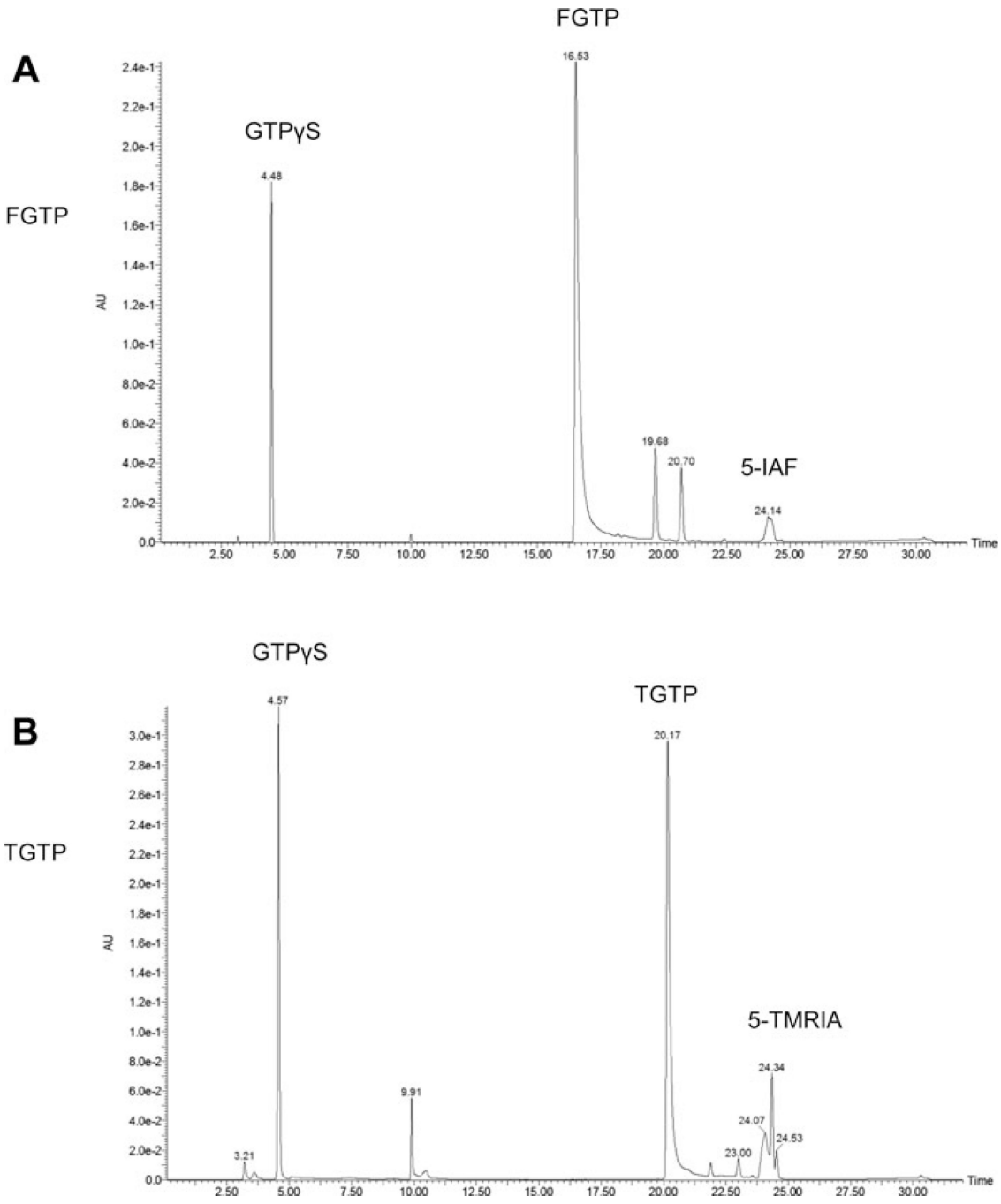


Figure 2.

Reverse-phase HPLC analysis. Reverse-phase C18 analytical HPLC was performed on starting materials and on reactions mixtures, measuring absorbance at 254 nm. (A) GTPγS only, (B) 5-IAF only, (C) 5-TMRIA only, (D) Presence of FGTP after combining GTPγS, and 5-IAF in a 3:1 ratio (E) Presence of TGTP after combining GTPγS and 5-TMRIA in a 3:1 ratio.

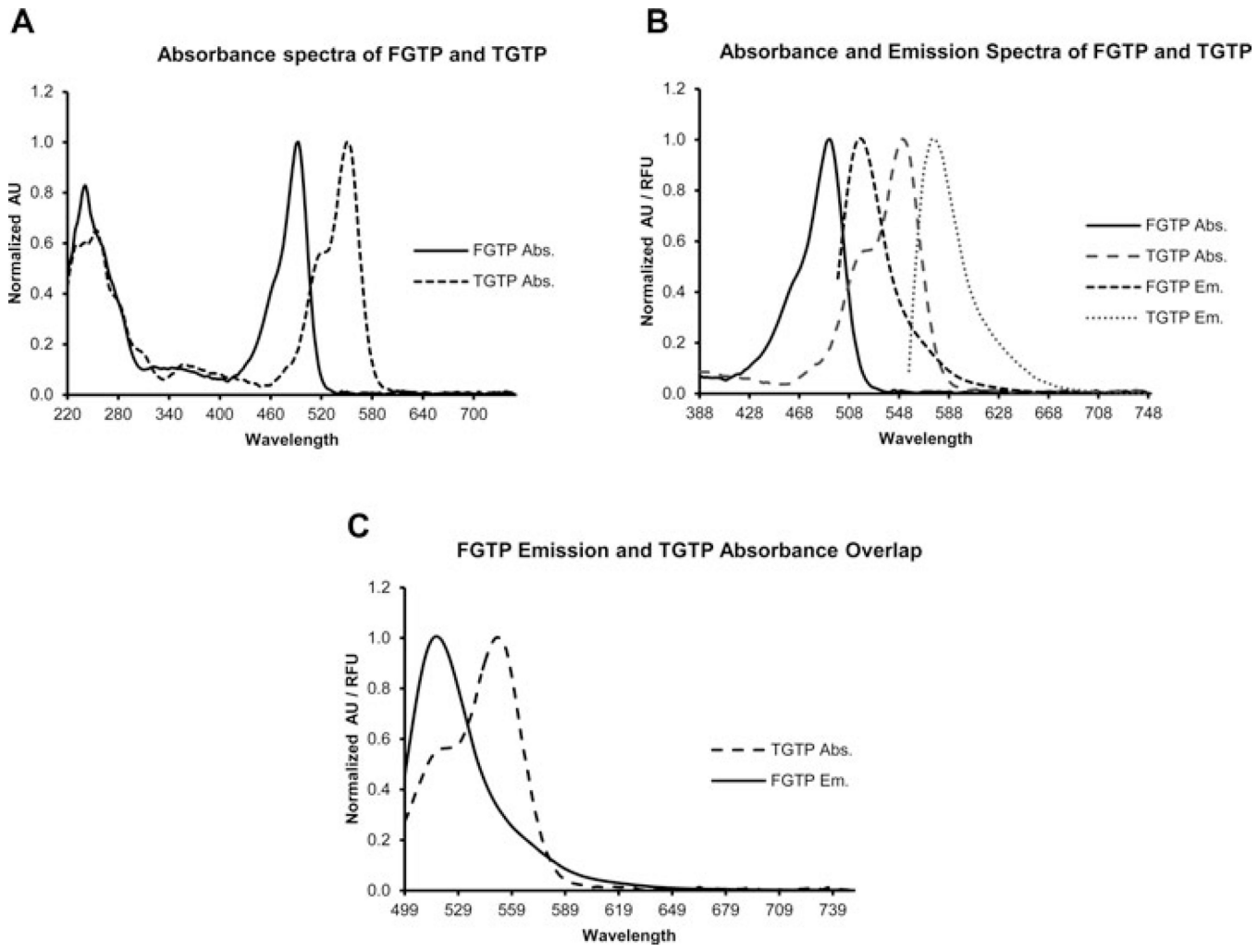


Figure 3.

Absorbance and emission spectra of FGTP and TGTP. (A) The successful conjugation of GTPγS with 5-IAF and 5-TMRIA was assessed by measuring absorbance of FGTP and TGTP, identifying peaks for the nucleotide and the fluorophore. (B) Graphs displaying the absorbance and emission spectral properties of the fluorophores. (C) Spectral overlap between FGTP emission and TGTP absorbance.

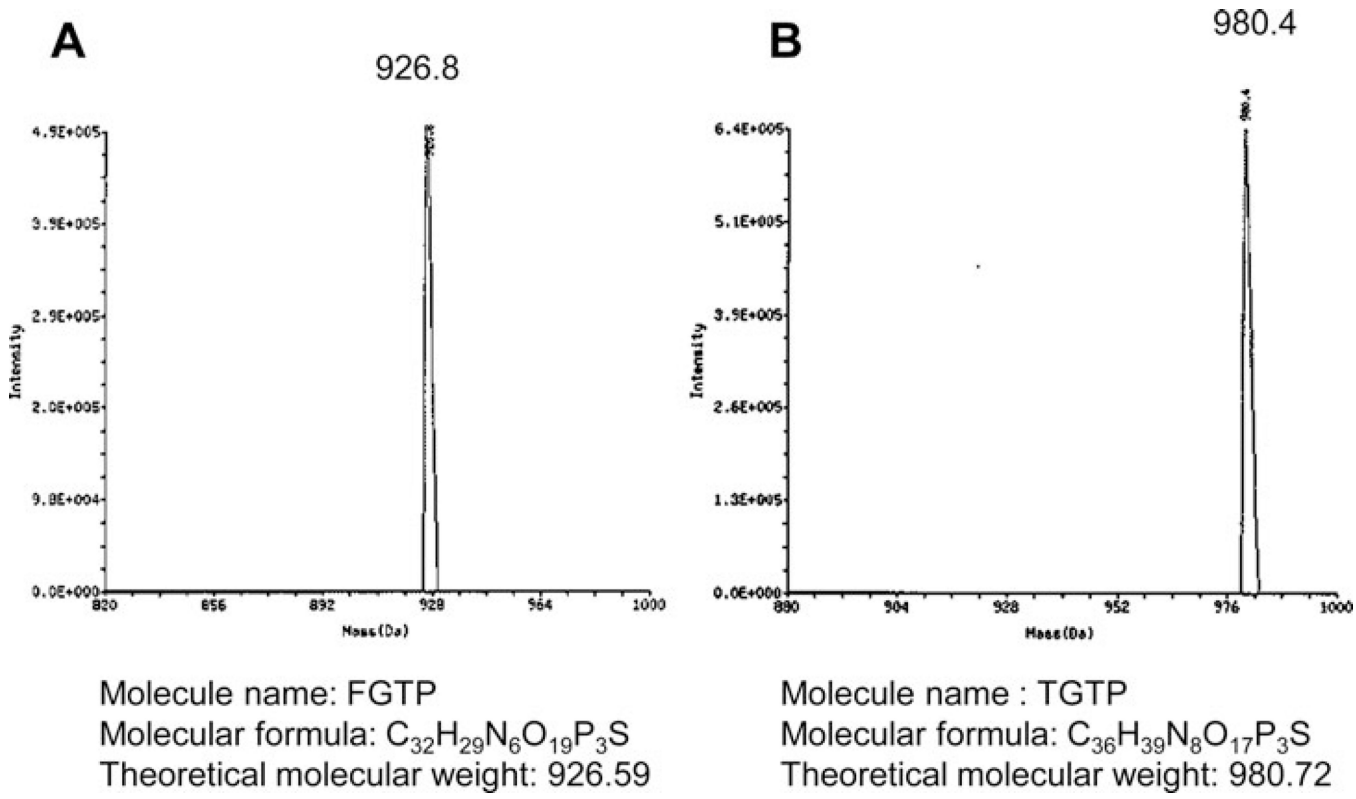
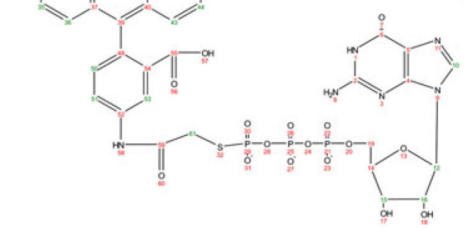


Figure 4.

Electrospray ionization mass spectrometry analysis. ESI-MS analysis revealed the presence of two new purified compounds (A) FGTP and (B) TGTP.

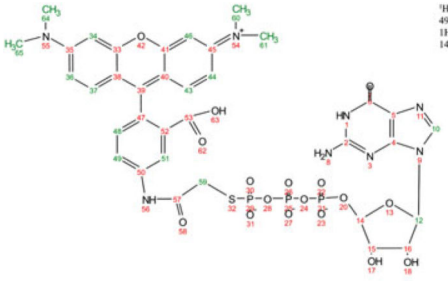
A



^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.04 (d, J = 2.2 Hz, 1H, 53, 53), 8.03 (s, 1H, 10, 10), 7.63 (dd, J = 8.2, 2.2 Hz, 1H, 51, 51), 7.24 – 7.22 (m, 1H, 36, 36), 7.13 (d, J = 9.2 Hz, 1H, 43, 43), 7.02 (d, J = 8.2 Hz, 1H, 50, 50), 6.68 (s, 1H, 33, 46, 33, 46), 6.68 – 6.66 (m, 2H, 35, 35), 6.62 (dd, J = 9.3, 2.3 Hz, 1H, 44, 44), 5.71 (d, J = 5.5 Hz, 1H, 12, 12), 4.43 (t, J = 5.3 Hz, 1H, 16), 4.39 (dd, J = 5.1, 3.8 Hz, 1H, 15), 3.82 (d, J = 14.2 Hz, 2H, 61, 61).

	Name	Shift	Range	H's	Integral	Class	J's
1	61 (d)	3.82	3.83 .. 3.80	2	1.72	d	14.21
2	53 (d)	8.04	8.04 .. 8.04	1	1.03	d	2.18
3	51 (dd)	7.63	7.65 .. 7.61	1	1.03	dd	2.23, 8.21
4	50 (d)	7.02	7.03 .. 7.00	1	1.00	d	8.17
5	44 (dd)	6.62	6.63 .. 6.61	1	1.03	dd	2.28, 9.25
6	43 (d)	7.13	7.14 .. 7.11	1	0.97	d	9.23
7	36 (m)	7.23	7.24 .. 7.22	1	0.84	m	
8	35 (m)	6.67	6.68 .. 6.66	2	1.89	m	
9	33,46 (s)	6.68	6.68 .. 6.68	1	1.18	s	
10	16 (t)	4.43	4.44 .. 4.41	1	0.92	t	5.34, 5.34
11	15 (dd)	4.39	4.40 .. 4.37	1	0.95	dd	3.85, 5.08
12	12 (d)	5.71	5.72 .. 5.69	1	1.05	d	5.48
13	10 (s)	8.03	8.03 .. 8.03	1	0.97	s	

B



^{1}H NMR (600 MHz, Deuterium Oxide) δ 8.14 (d, J = 2.2 Hz, 1H, 51), 8.05 (s, 1H, 10), 7.84 (dd, J = 8.2, 2.2 Hz, 1H, 49), 7.43 (d, J = 8.2 Hz, 1H, 48), 7.20 (d, J = 9.5 Hz, 1H, 37), 7.15 (d, J = 9.5 Hz, 1H, 43), 6.80 (dd, J = 9.5, 2.5 Hz, 1H, 36), 6.73 (dd, J = 9.5, 2.5 Hz, 1H, 44), 6.35 (s, 1H, 34), 6.28 (s, 1H, 46), 5.72 (d, J = 6.1 Hz, 1H, 12), 3.87 (d, J = 14.8 Hz, 2H, 59), 3.11 (s, 7H, 64, 65), 3.09 (s, 6H, 60, 61).

	Name	Shift	Range	H's	Integral	Class	J's
1	64,65 (s)	3.11	3.13 .. 3.10	7	6.65	s	
2	60,61 (s)	3.09	3.10 .. 3.07	6	6.41	s	
3	59 (d)	3.87	3.90 .. 3.84	2	1.57	d	14.81
4	51 (d)	8.14	8.15 .. 8.13	1	1.05	d	2.21
5	49 (dd)	7.84	7.86 .. 7.83	1	1.00	dd	2.18, 8.16
6	48 (d)	7.43	7.46 .. 7.41	1	1.03	d	8.16
7	46 (s)	6.28	6.31 .. 6.25	1	1.19	s	
8	44 (dd)	6.73	6.76 .. 6.71	1	1.05	dd	2.52, 9.48
9	43 (d)	7.15	7.18 .. 7.13	1	1.08	d	9.47
10	37 (d)	7.20	7.24 .. 7.18	1	1.10	d	9.46
11	36 (dd)	6.80	6.83 .. 6.77	1	1.10	dd	2.47, 9.47
12	34 (s)	6.35	6.38 .. 6.32	1	1.00	s	
13	12 (d)	5.72	5.73 .. 5.71	1	1.00	d	6.10
14	10 (s)	8.05	8.06 .. 8.04	1	1.04	s	

Figure 5.

Assignment of ^{1}H NMR signals of FGTP and TGTP. The chemical structures and ^{1}HNMR data are given for (A) FGTP and (B) TGTP.

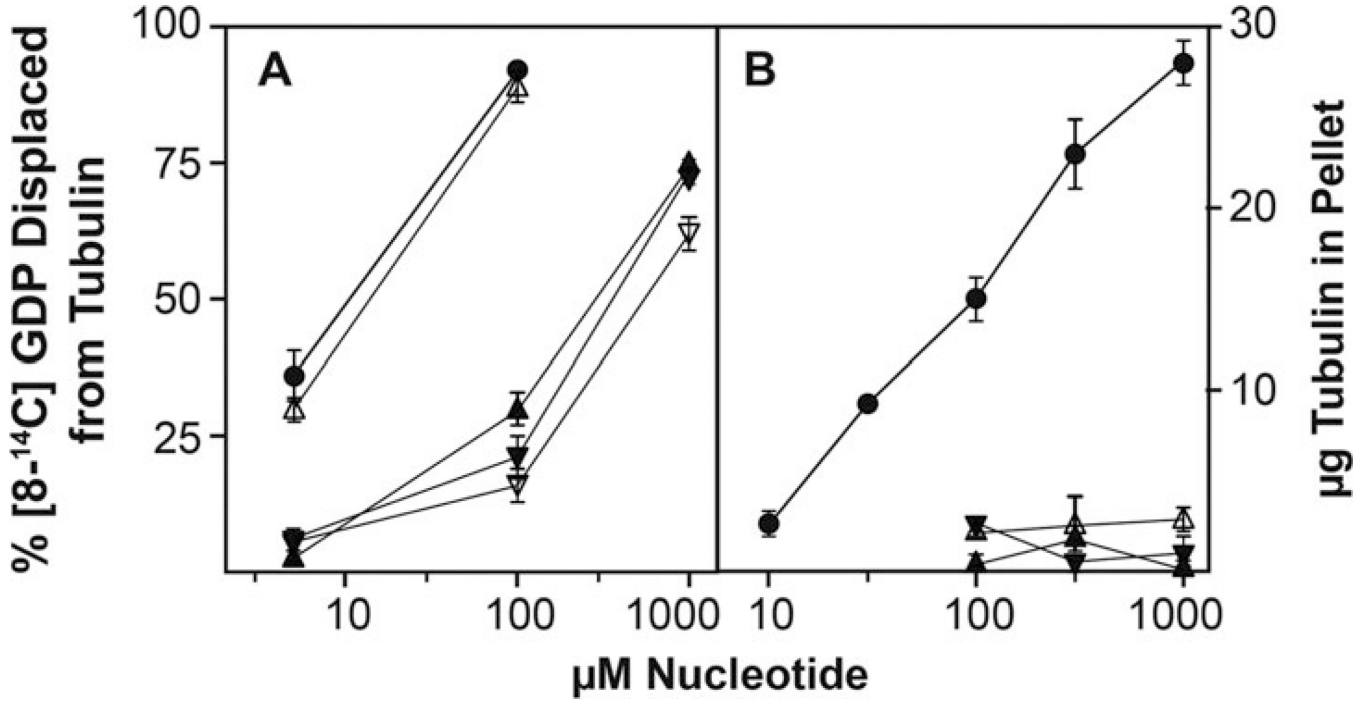


Figure 6.

FGTP and TGTP interaction with tubulin. (A) [8-^{14}C]-GDP displacement from tubulin by various guanine nucleotides. (B) Tubulin polymerization as measured by microtubule pellet formation after centrifugation. - GTP, - GTPγS, - GDPβS, - FGTP, - TGTP.