

Effects of Purinenucleotide Analogues on Microtubule Assembly

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ABSTRACT. This minireview summarizes the syntheses of various purinenucleotide analogues and their effects on microtubule (Mt) assembly. 27 analogues were so far synthesized and, together with 3 analogues commercially available (ITP, XTP and dGTP), their effects on Microtubule assembly were investigated. The positions C2, C6, C8, and ribose moiety of purine nucleotides were modified or substituted. It was found that the microenvironments of the purine base and ribose moiety are important for the nucleotides to support Mt assembly. Introduction of amino group into position C2 of ATP, formation of 2-amino ATP, caused Mt assembly substantially. 2-Amino deoxy ATP and deoxy GTP are more potent than GTP in supporting assembly. The introduction of reactive thiol group into C6 (6-SH-GTP) largely reduces the activity of the analogue to support assembly. However, sequestering reactivity of the thiol group by association with methyl group largely recovers the ability of the analogue to promote assembly. Free rotation of the glycosidic linkage was found to be also inevitable in promoting assembly, as the introduction of sulfur atom between C8 of the purine base and C2' of the ribose moiety (formation of 8,2'-S-cyclo purine nucleotides) caused total inhibition. Purinenucleoside triphosphate supports assembly better than GTP but the deoxy-type analogues are totally inhibitory. 2-Amino-8-hydroxy ATP and other analogues support assembly much better than does GTP. However, their diphosphate analogues are totally incapable of supporting assembly. Introduction of a bulky fluorescent probes into C3' can be made to visualize the fluorescent signal in assembled Mts. Together with the suggestions proposed from electron crystallography of zinc-induced tubulin sheets, interactions of the purine base and ribose moieties with surrounding amino acid residues are discussed.

Key words: purinenucleotide analogues/microtubule assembly/tubulin

Introduction

Two binding sites for GTP, one exchangeable and the other non-exchangeable sites, on the $\alpha\beta$ tubulin heterodimer have well been known (5, 14, 30) and the exchangeable site has been shown to be localized on β -tubulin (4, 22).

The role of guanine nucleotides on microtubule (Mt) assembly has long been discussed on the assumption that the conformation of the tubulin heterodimer will

be maintained by GTP for tubulin polymerization. Non-hydrolyzable GTP analogues have been reported to support tubulin polymerization (1, 22, 24, 31), and some analogues of GTP were shown to react with tubulin heterodimer (3, 32).

Recent electron crystallographic data on zinc-induced tubulin sheets suggest the positioning of GTP in the $\alpha\beta$ tubulin heterodimer and interactions of the phosphate, ribose and base moieties with surrounding amino acid residues (23).

Various analogues of GTP would further help our understanding on the tubulin heterodimers concerning the role of purinenucleotides in polymerization. A few reports have been published describing the effect of GTP analogues, ribose moiety of which has been modified (5–7), and some modification of the purine base which supported Mt assembly although it was under non-physiological conditions (3). Recently, various analogues of purinenucleotide were synthesized and their

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Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; EGTA, ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HPLC, high performance liquid chromatography; MAPs, microtubule-associated proteins; Mts, microtubules; PEP, paper electrophoresis; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); TEAB, triethylammonium carbonate; TLC, thin layer chromatography. For abbreviations of the purinenucleotide analogues, see Table I–IV.

effects on Mt assembly were reported (21). This may suggest the nature of microenvironments surrounding the base and sugar moieties of the nucleotide in the tubulin heterodimer.

Syntheses of various purinenucleotide analogues

Syntheses of base-modified analogues of purinenucleoside 5'-triphosphate

In order to investigate tubulin-nucleotide interaction in Mt assembly, more than 21 base-modified analogues of purinenucleoside 5'-triphosphate (2, 19) including dGTP (20) and 8,2'-S-cyclo analogues of purinenucleoside 5'-triphosphate (12, 18) were synthesized.

Base-modified analogues of guanosine and 8,2'-S-cyclopurinenucleoside were converted into the corresponding purinenucleoside 5'-monophosphate derivatives by phosphorylation with phosphoryl chloride in trimethylphosphate at 0–4°C for 18 h (Fig. 1), followed by addition of 0.5 M sodium bicarbonate. After neutralization, the mixture was applied to a column of DEAE cellulose and eluted with a linear gradient of 0–0.3 M trimethyl ammonium carbonate (TEAB). The fractions at the main peak were pooled and evaporated.

Deoxyguanosine derivatives were phosphorylated with cyanoethylphosphate in the presence of dicyclohexylcarbodiimide (DCC) in pyridine at 25°C for 18 h. The crude purinedeoxynucleoside 5'-cyanoethylphosphate was purified by DEAE-Sephadex A-25 column

chromatography. A linear gradient of 0–0.1 M TEAB was used and the main fractions were pooled and evaporated. To the residues concentrated NH_4OH was added and stirred for 1 h, followed by evaporation of the solution to dryness. Purinenucleoside 5'-monophosphate (Fig. 1) was identified by hydrolysis with snake venom 5'-nucleotidase to the corresponding nucleoside on paper electrophoresis (PEP).

Purinenucleoside 5'-monophosphate was converted to the intermediate following by two synthetic methods. A: Purinenucleoside 5'-monophosphate was converted into purinenucleoside 5'-phosphorimidazolidate (Fig. 1) by 1,1-carbonylimidazole in dimethylformamide (DMF). The reaction mixture was stirred for 5 h at 25°C, then treated with methanol at 25°C for 0.5 h. The mixture was used in the following phosphorylation step without further purification. B: Purinenucleoside 5'-monophosphate was converted into purinenucleoside 5'-phosphormorpholidate (Fig. 1) with morpholine and DCC in *tert*-butanol (17). The reaction mixture was refluxed for 8 h and evaporated to dryness. Water was added to the residue and then cyclohexylurea formed was filtered off and evaporated until dryness. The intermediate 5'-phosphormorpholidate was also used for the next phosphorylation step without further purification.

Purinenucleoside 5'-phosphorimidazolidate or 5'-phosphormorpholidate was phosphorylated to the corresponding 5'-triphosphate (Fig. 1) by tri-*n*-butyl ammonium pyrophosphate in DMF for 16 h at 25°C.

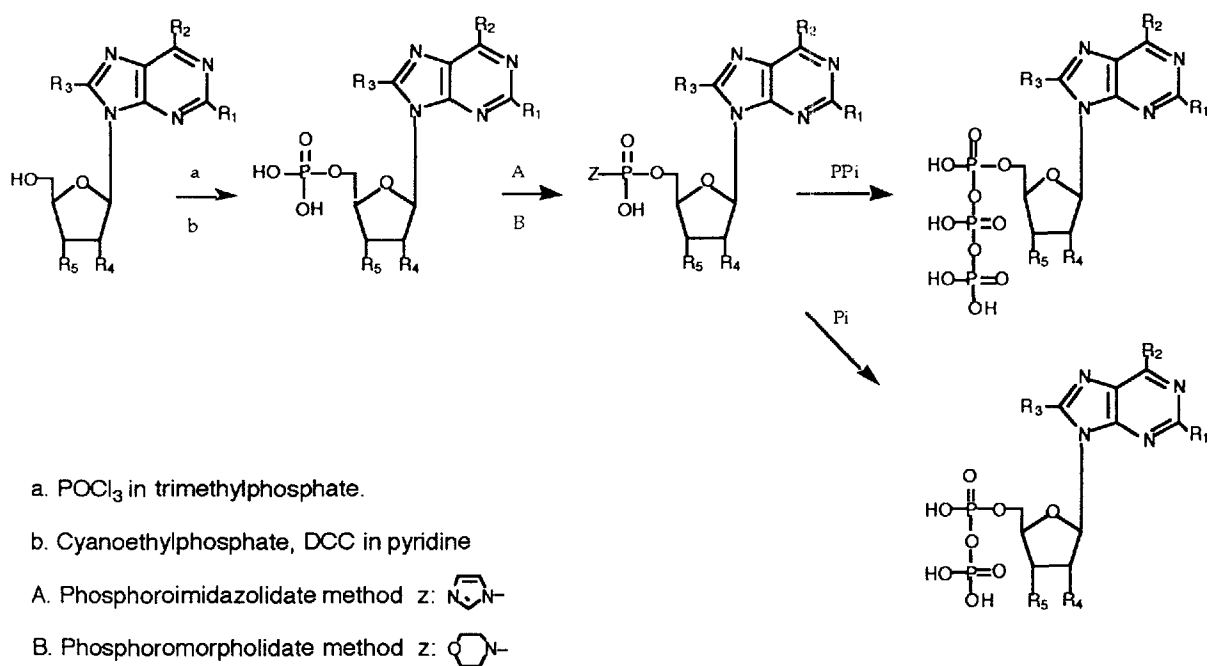


Fig. 1. Synthetic pathway of purinenucleotide analogues.

Purinenucleoside 5'-phosphoromorpholidate (Fig. 1) was dried by coevaporation with pyridine. Separately, a solution of 85% orthophosphoric acid and tri-*n*-butylamine in pyridine was also rendered anhydrous by coevaporation. The two pyridine solutions were mixed and stirred for 1 h and kept standing for 48 h at 30°C.

Synthesis of ribose-modified fluorescent analogues of purinenucleoside 5'-triphosphate

Among various nucleotide analogues, fluorescent ones are useful because of the high sensitivity to small

No.	R ₁	R ₂	R ₃	R ₄	R ₅	Abbreviation	Efficiency to affect Mt assembly
1	NH ₂	NH ₂	H	OH	OH	n2ATP	P
2	O	NH ₂	H	OH	OH	iso-GTP	I
3	NH ₂	NH ₂	H	H	OH	n2dATP	HP
4	H	O	H	OH	OH	ITP	HP
5	C ₈ H ₅ NH	NH ₂	H	OH	OH	2-Anil-ATP	P
6	O	O	H	OH	OH	XTP	SI
7	NH ₂	O	H	H	OH	dGTP	P
8	NH ₂	C ₆ H ₅ NH	H	H	OH	6-Anil-n2dPuTP	P
9	NH ₂	O(CH ₂ CH ₂) ₂ NH	H	H	OH	6-Mor-n2dPuTP	I
10	NH ₂	C ₆ H ₁₁ NH	H	H	OH	6-CHex-n2dPuTP	P
11	H	NH ₂	COOH	OH	OH	8-COOH-ATP	HI
12	H	EtCO ₂ CH ₂	H	OH	OH	6-EtCO ₂ CH ₂ -PuTP	P
13	NH ₂	SH	H	OH	OH	6-SH-GTP	HI
14	NH ₂	CH ₃ S	H	OH	OH	6-CH ₃ S-GTP	P
15	NH ₂	NH ₂	OH	OH	OH	8-OH-n2ATP	HP
16	NH ₂	O	Br	OH	OH	8-Br-GTP	P
17	NH ₂	NH ₂	Br	OH	OH	8-Br-n2ATP	SI
18	H	H	H	OH	OH	PuTP	HP
19	NH ₂	H	H	OH	OH	n2PuTP	HP
20	H	H	H	H	OH	dPuTp	HI
21	H	H	H	H	H	ddPUTP	HI

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Table II. STRUCTURE AND ABBREVIATION OF 8,2'-S-CYCLO GTP ANALOGUES AND THEIR EFFICIENCY TO AFFECT MT ASSEMBLY

No.	R ₁	R ₂	R ₃	Abbreviation	Efficiency to affect Mt assembly
22	NH ₂	NH ₂	OH	8,2'-S-n2ATP	HI
23	H	NH ₂	OH	8,2'-S-ATP	HI
24	NH ₂	O	OH	8,2'-S-GTP	HI

charges in environment as well as requirement of a low concentration sufficient to obtain meaningful results.

According to the procedure reported by Hiratsuka (9), 3'-*O*-(*N*-methylantraniloyl) GTP (Mant GTP), 3'-*O*-(*N*-methylantraniloyl)8-bromo GTP (Mant-8-Br-GTP) and 3'-*O*-(*N*-methyl-antraniloyl)2,6-diamino-8-hydroxy ATP (Mant-8-OH-n2 ATP) were synthesized (Fig. 2). Briefly describing, nucleotide was dissolved in a minimum amount of water. The pH was adjusted to 9.6 with NaOH, followed by addition of *N*-methylisatoic anhydride and stirring for 2 h. After comple-

tion of the reaction, the pH of the reaction mixture was adjusted to 7.0 with 1 N HCl and subjected to column chromatography on Sephadex LH-20. Elution was carried out by water and the fraction containing the product was checked by TLC on silica gel. The peak fraction of the fluorescent analogue was evaporated to dryness. The residue was dissolved in a minimum amount of water and methanol, converted to the sodium salt and reevaporated to dryness. The purity of the product was checked by PEP and HPLC and confirmed by the elementary analysis. The structures and abbreviations

Table III. STRUCTURE AND ABBREVIATION OF GDP ANALOGUES AND THEIR EFFICIENCY TO AFFECT MT ASSEMBLY

No.	R ₁	R ₂	R ₃	R ₄	R ₅	Abbreviation	Efficiency to affect Mt assembly
25	NH ₂	NH ₂	OH	OH	OH	8-OH-n2ADP	I
26	NH ₂	NH ₂	H	OH	OH	n2ADP	I
27	NH ₂	NH ₂	H	H	OH	n2dADP	I

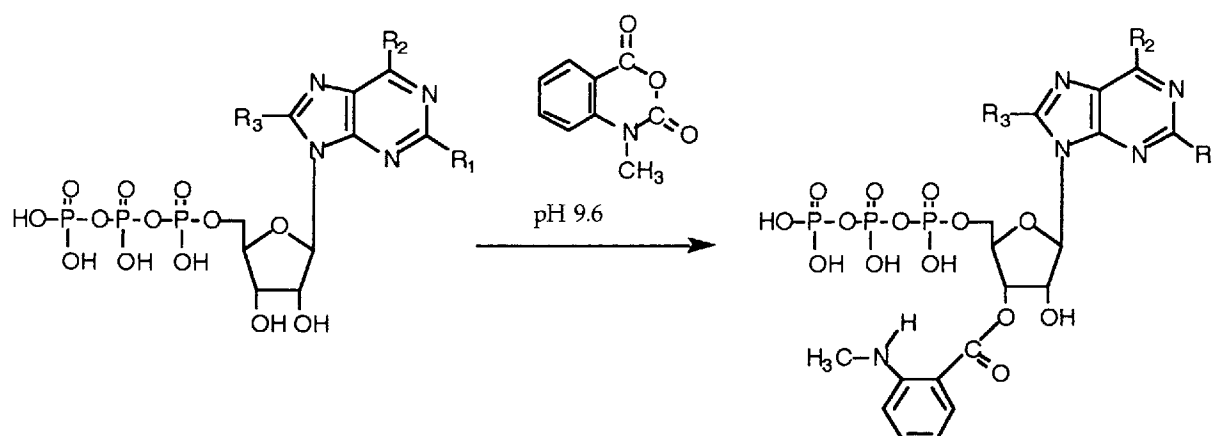


Fig. 2. Synthesis of Mant GTP analogues.

of Mant GTP analogues are shown in Table IV. The absorption and fluorescence spectrum data of the analogues are shown in Table V.

Effects of purinenucleotide analogues on microtubule assembly

Table I-IV summarizes the efficiency of the analogues in affecting Mt assembly (21).

Modification of position C2 of the purine base

It has been well known that ATP does not support Mt assembly, neither ATP nor ADP associates with Mts (15). However, the addition of amino group to position C2 of the ATP purine base (n2ATP) was newly

found to largely enhance the property of the analogue to cause Mt assembly. More than half the initial growth rate and steady state of Mt assembly in the GTP standard assembly system were attained by the substitution. Mts assembled in n2ATP-containing buffer were indistinguishable with those assembled in the GTP standard buffer as observed by electron microscopy. When C2 amino group and C6 oxo group are exchanged (formation of isoGTP), it became to fail to support assembly. The substitution of the amino group at position C2 of GTP with hydrogen, primary and secondary amines and oxo group resulted in the following order in promoting assembly, hydrogen (ITP) > primary alkyl amino group (GTP) > secondary aryl amino group (2-Anil-ATP) > oxo group. The possibility was consid-

Table IV. STRUCTURE AND ABBREVIATION OF MANT GTP ANALOGUES AND THEIR EFFICIENCY TO AFFECT MT ASSEMBLY

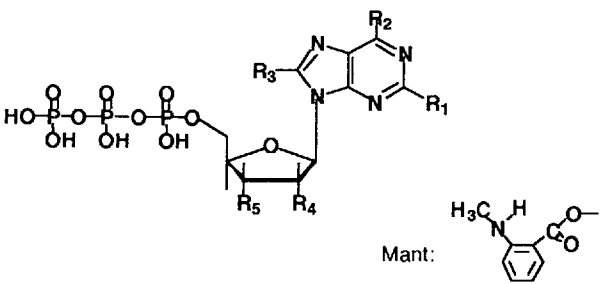
							Efficiency to affect Mt assembly
No	R ₁	R ₂	R ₃	R ₄	R ₅	Abbreviation	
28	NH ₂	O	H	OH	Mant	Mant-GTP	SI
29	NH ₂	O	Br	OH	Mant	Mant-8-Br-GTP	SI
30	NH ₂	NH ₂	OH	OH	Mant	Mant-8-OH-n2ATP	HI

Table V. ABSORPTION AND FLUORESCENCE PROPERTIES OF METHYLANTHRANILLOYL (MANT) GTP ANALOGUES

Compound	Absorption spectra λ_{max} (nm)**		Fluorescence spectra λ_{max} (nm)***
Mant-GTP*	252.5	356.5	443
Mant-8-Br-GTP	256	356.5	437
Mant-8-OH-n2ATP	249	291 356	432

* According to Hiratsuka (9), absorption maxima for Mant GTP were observed at 252 nm and 350 nm in 50 mM Tris-HCl (pH 8.0) and fluorescence maxima were at 442 nm in H₂O and 432 nm in ethanol when excited at 330 nm and 350 nm, respectively.

** Absorption spectra were measured in 50 mM Tris-HCl (pH 8.0).

*** Fluorescence spectra were measured in 80% ethanol. Excitation was performed at 355 nm for all the Mant GTP analogues.

ered that some hydrogen bonding through hydrogen atoms of amino group at position C2 with nearby oxygen or nitrogen atoms in β -tubulin molecule might be involved in the maintenance of a conformation favorable for tubulin polymerization.

6-Substituted dGTP analogue

Hamel and Lin (5, 7) demonstrated that deoxy GTP promoted assembly better than GTP. This was confirmed by Muraoka *et al.* (21) and similar phenomena were shown, newly synthesized n2dATP stimulated assembly much better than n2ATP, that is, deoxy-type of GTP or n2ATP is more potent in promoting Mt assembly than the hydroxy-type nucleotides. The experiments performed by Muraoka *et al.* (21) suggest that positions C6 and C2 of the purine base seem to be important for the nucleotide to support Mt assembly. In their experiment, 6-Anil-n2dPuTP and 6-Mor-n2dPuTP were inhibitory. On the other hand, the substitution of oxo group with amino group (n2dATP), or cyclohexyl group (6-CHex-n2dPuTP) largely supported Mt assembly. The substitution of oxo group with bulky cyclohexyl group suppressed the initial velocity of polymerization but the steady state was higher than that monitored in the standard GTP buffer solution. Furthermore, the introduction of bulky group like ethoxycarbonyl group (6-EtCOOCH₂-PuTP) at position 6 supported assembly to some extent.

When the oxo group at the C6 of GTP was substituted with thiol (6-SH-GTP), it resulted in almost total failure in promoting assembly. Electron microscopy revealed that only ring structures without Mts were observed. This result is inconsistent with those reported by Fishback and Yarbrough (3, 32), probably due to the presence of glycerol (25% w/v, about 3.4 M) in their experiments. In the presence of 4 M glycerol, tubulin assembles into Mts even in the absence of GTP (27) or in the presence of GDP (15). The inhibition of assembly by the introduction of thiol was suggested to not be

due to the crosslinking between the introduced thiol at the C6 of the nucleotide base and the cysteine residue 12 in β -tubulin involved in photocrosslinking (28), because the total inhibition occurred even in the presence of reducing reagent.

When the reactivity of thiol group was sequestered by burdening methyl group (forming 6-CH₃ S-GTP), namely, the introduction of a hydrophobic group, it largely resumed the activity to promote Mt assembly (21). This may suggest that hydrophobic microenvironment is needed around C6 to support assembly. The introduction of amino group at the position C2 and hydroxy group at the position C8 of ATP, forming 8-OH-n2ATP, supported assembly much more than did GTP. This assembly system was also inhibited by Mt toxins such as colchicine or podophyllotoxin at a sub-stoichiometric concentration.

8-Substituted GTP analogues

The modification of the position C8 of the purine base substantially affects the activities of the nucleotide analogues. The substitution of hydrogen atoms at the position C8 with hydroxy group (8-OH-n2ATP) or bromo group (8-Br-GTP), promotes assembly better than does GTP or 8-Br-n2ATP.

8,2'-S-Cyclo GTP analogues

The blocking of the rotation of the glycosidic linkage of GTP was performed by Muraoka *et al.* (21). They synthesized 8,2'-S-cyclo GTP analogues (8,2'-S-n2ATP, 8,2'-S-ATP and 8,2'-S-GTP), which were found to be almost totally inhibitory in Mt assembly. This result seems to indicate that the rotation around the glycosidic linkage is inevitable for the binding of GTP to β -tubulin to sustain the heterodimer conformation favoring polymerization into Mts. However, 8,2'-anhydro-8-mercapto-purinenucleosides have a fixed high-anti conformation (8, 11–13, 29) due to the anhydro linkage between the base C8 position and the C2' position of the sugar moiety. Therefore, the possibility is not excluded that distortion of the nucleotide conformation due to the high-anti conformation caused suppression of the assembly promoting ability of GTP.

Purinenucleoside 5'-triphosphate analogues

Purinenucleoside 5'-triphosphate (PuTP) supported Mt assembly better than did GTP. An experiment was performed to see whether or not deoxygenization of hydroxy group of ribose moiety affect the assembly-promoting ability of the analogues (21). Deoxy- and dideoxy-type of the analogues (dPuTP and ddPuTP) were shown to lose the Mt assembly promoting activity almost completely. Since deoxy GTP promotes assembly better than does GTP, the above results reinforce the

idea that the position C2 of GTP is important for supporting assembly.

GDP analogues

Irrespective of the high ability to promote Mt assembly of the triphosphate-type analogues (8-OH-n2ATP, n2ATP and n2dATP), their diphosphates almost completely failed to support assembly (21). Since Mt assembly is accompanied by dephosphorylation of the E-site GTP on β -tubulin, the E-site needs to be in the triphosphate form before assembly (26). It is clear that guanosine triphosphate promotes assembly, consistent with the result reported by Hamel *et al.* (6) which demonstrated that guanosine tetraphosphate does not support assembly.

Mant GTP

The introduction of methylantraniloyl (Mant) group into the position C3' favors fluorescent labeling of tubulin (9, 10). Mant-GTP and Mant-8-Br-GTP substantially supported assembly, but Mant-8-OH-n2ATP did not. Mant-GTP or Mant-8-Br-GTP produced many Mts fluorescently labeled, corroborating the work done by Hiratsuka (10).

The number of protofilaments consisting of Mts assembled from tubulin saturated with Mant-8-Br-GTP was shown to be 14 (21), which is consistent with the results reported by Pierson *et al.* (25). The introduction of a bulky group like methylantraniloyl (Mant) group into ribose moiety of GTP does not largely alter the arrangement of the microtubule lattice. The Mant-derivative of GTP may be useful in determining the affinity of non-labeled nucleotide analogues for nucleotide-binding proteins as described by Mills and Richter (16).

Electron crystallography of zinc-induced tubulin sheets (23) suggested that the possible amino acids surrounding the guanine base may be tyrosine 224 and asparagine 206 and 228. Therefore, a possibility can be presented that the formation of hydrogen bond between the oxygen atom of asparagine 206 or 228 and the position C2 amino group, hydrophobic interaction between the position C6 and the tyrosine 224 and the positioning of triphosphate might be inevitable for the maintenance of the conformation of the tubulin heterodimer favorable for Mt assembly.

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