Stereospecific Synthesis of Guanosine 5′-O-(1,2-Dithiotriphosphates)

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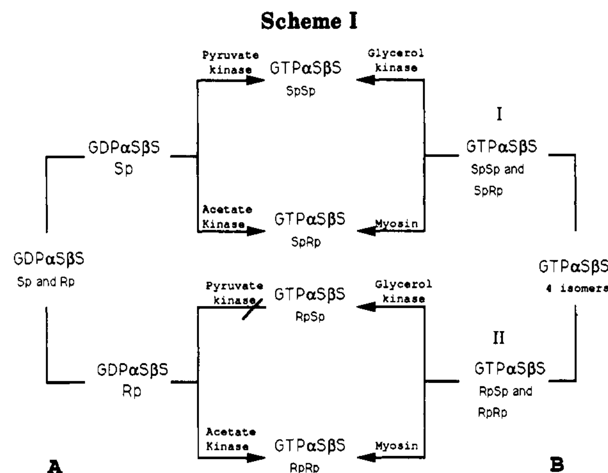
The synthesis of the four diastereomers of guanosine 5′-O-(1,2-dithiotriphosphate) by two different approaches is described. 2′,3′-Diacetylguanosine is phosphitylated with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one to form 2, which upon reaction with S-2-cyanoethyl phosphorothioate and sulfur forms 3. Subsequent hydrolysis of this intermediate produces the two diastereomers of guanosine 5′-O-(1,2-dithiodiphosphate) (GDPαSβS, 4). These can be phosphorylated by both acetate or pyruvate kinases. The R_pR_p as well as the S_pR_p diastereomers of guanosine 5′-O-(1,2-dithiotriphosphate) (GTPαSβS, 5) can be obtained by reaction of (R_p)- or (S_p)-GDPαSβS, respectively, with acetate kinase. Pyruvate kinase only accepts (S_p)-GDPαSβS as substrate and therefore only (S_pS_p)-GTPαSβS is available by this route. All four diastereomers of GTPαSβS can be synthesized by the second approach, in which 2 is reacted with thiopyrophosphate to produce a mixture of the diastereomers of GTPαSβS and of guanosine 5′-O-(1,3-dithiotriphosphate) (GTPαSγS). The latter is removed by selective hydrolysis and subsequent chromatography. The four diastereomers of GTPαSβS can be separated by HPLC according to their configuration at Pα into two groups consisting of the S_pS_p/S_pR_p and the R_pS_p/R_pR_p diastereomers. Glycerol kinase selectively hydrolyzes the diastereomers with the R_p configuration at Pβ, thus making (S_pS_p)- and (R_pS_p)-GTPαSβS available from the two groups. Myosin is stereospecific for reaction with diastereomers with the S_p configuration at Pβ, allowing the isolation of the S_pR_p and R_pR_p diastereomers. ^{31}P NMR spectroscopy of the Cd^{2+} salts of the four diastereomers reveals two groups of complexes, which differ in their Pαβ coupling constants which can be related to the arrangement of the guanosine and the γ-phosphate as either cis or trans in the six-membered cyclic complex.

Nucleoside phosphorothioates have found wide application in biochemistry and molecular biology.[1,2] Nucleoside 5′-O-(1-thiotriphosphates) and nucleoside 5′-O-(2-thiotriphosphates) exist as pairs of diastereomers designated R_p and S_p according to their configuration around α-phosphorus atom. Many enzymes show a strong preference for one or the other diastereomer and often this stereoselectivity can be reversed by a change from hard to soft metal ion.[1,3] This stereoselectivity can be used as an analytical tool to differentiate between individual enzymes within a given family of enzymes. Of particular interest in this area are the guanine nucleotide-binding proteins (G-proteins), which are involved in signal transduction pathways (for a review, see ref 4). Thus, there is considerable preference for one of the diastereomers of guanosine 5′-O-(1-thiotriphosphate) (GTPαS) in activating the olfactory-specific G-protein,[5] whereas little difference is seen in their interaction with transducin, the G-protein implicated in vision.[6] The two proteins also differ in their specificity for the diastereomers of guanosine 5′-O-(2-thiotriphosphate) (GTPβS). The olfactory-specific G-protein shows no preference for either diastereomer, whereas transducin has a stronger affinity for the S_p isomer. The G-protein responsible for the oscillatory release of Ca^{2+} in mast cells is, on the other hand, preferentially activated by (R_p)-GTPβS.[7] Further examples include the interaction of GTPβS with ras oncogene p21 and the elongation factor EF-Tu.[8] These examples make it attractive to try to use the four diastereomers of the doubly phosphorothioate-substituted guanosine 5′-O-(1,2-dithiotriphosphate) (GTPαSβS) to further elucidate differences between various G-proteins. We describe here the first synthesis of these compounds and their interaction with soft metal ions.

Results and Discussion

Two strategies followed for the synthesis of the four individual diastereomers of GTPαSβS are presented (Scheme I). One approach consists of the chemical synthesis of the two diastereomers of guanosine 5′-O-(1,2-dithiodiphosphate) (GDPαSβS) and the introduction of the γ-phosphate by stereospecific phosphorylations catalyzed by acetate and pyruvate kinase (route A, Scheme I). These enzymes have been shown earlier to be capable of phosphorylating guanosine 5′-O-(2-thiodiphosphate) (GDPβS).[9] The second approach consists of the chemical synthesis of the four diastereomers of GTPαSβS and their resolution into individual diastereomers by chromatographic separation and selective enzymatic dephosphorylation (route B, Scheme I). The starting material for route A is GDPαSβS (4). Preliminary attempts to synthesize GDPαSβS from guanosine 5′-phosphorothioate by the Michelson procedure with S-cyanoethyl thiophosphate produced only moderate yields although this reaction

Scheme I



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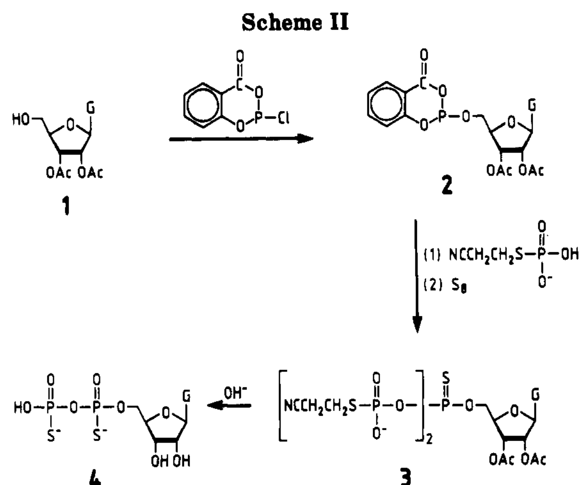
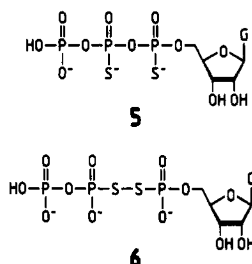
Scheme II



proceeds reasonably well with the adenosine derivative.[10] However, it has been demonstrated before that the Michelson method is very inefficient with guanosine derivatives as exemplified by the synthesis of GDPαS.[9] Therefore, a method based on previously published procedures for the synthesis of nucleoside 5′-O-thiotriphosphates[11] and nucleoside 5′-(1,3-dithiotriphosphates)[12] via phosphite–phosphate mixed anhydrides was chosen (Scheme II). This synthesis makes the four diastereomers of GTPαSβS (5) easily accessible, starting from the commercially available 2′,3′-diacetylguanosine.



2′,3′-Diacetylguanosine (1) was phosphitylated with salicyl phosphorochloridite and the resulting 5′-phosphitylated derivative 2 was reacted with a 3-fold excess of S-cyanoethyl thiophosphate tri-n-butylammonium salt in anhydrous DMF. After oxidation with sulfur, the ^{31}P NMR spectrum of the reaction mixture revealed the presence of the branched triphosphate derivative 3. This spectrum consists of a triplet at 37.63 ppm (Pα), and two close doublets at 7.55 and 7.47 ppm (both Pβ) ($J_{\alpha\beta}$ = 33.6 Hz). This compound could be cleanly hydrolyzed to form 2′,3′-diacetylguanosine 5′-2-S-β-cyanoethyl 1,2-dithiodiphosphate), whose ^{31}P NMR spectrum shows signals at 43.08 ppm (d) (Pα) and 5.68 ppm (d) (Pβ) ($J_{\alpha\beta}$ = 36 Hz). Hydrolysis presumably occurs by attack on Pβ as shown by the reaction of 3 with morpholine, which results in the formation of the same product together with S-cyanoethyl thiophosphomorpholidate (23 ppm; s).

Removal of the cyanoethyl and acetyl groups from 3 resulted in the formation of GDPαSβS (4). This compound was characterized by its ^{31}P NMR spectrum with signals at 41.07 ppm (d) (Pα, S_p), 40.74 ppm (d) (Pα, R_p), and 33.80 ppm (d, Pβ) (J = 40.5 Hz). It was isolated as a 1:1 mixture of the two diastereomers after DEAE-Sephadex purification. The two diastereomers were easily separated by reverse phase HPLC. Occasionally small amounts of GTPαSγS were observed in this reaction when traces of S-cyanoethyl thiodiphosphate had been formed upon long storage of cyanoethyl thiophosphate in DMF.

The phosphorylation of GDPαSβS was performed by stereospecific enzymatic reactions with acetate and pyruvate kinase (Scheme I, route A). It has been shown earlier that in presence of Mg^{2+} acetate kinase catalyzes the phosphorylation of the pro-R phosphorothioate oxygen of GDPβS, whereas pyruvate kinase catalyzes the phosphorylation predominantly of the pro-S phosphorothioate oxygen.[9] The same stereoselectivity at the β-position was also found here for the enzymatic phosphorylation of the Mg salts of GDPαSβS by these two kinases. As acetate kinase was found to be nonstereospecific for the configuration at the α-position of GDPαSβS, the two diastereomers (S_pR_p)- and (R_pR_p)-GTPαSβS could be obtained from the two diastereomers of GDPαSβS by reaction with this enzyme.

However, pyruvate kinase showed a strong preference for the S_p configuration in the α-position so that by enzymatic phosphorylation of a mixture of diastereomers of GDPαSβS, the (S_pS_p)-GTPαSβS could be synthesized stereospecifically. The R_pS_p isomer is inaccessible by this route.

In the presence of Cd^{2+} reversal of specificity for the β-phosphorothioate has been demonstrated for the acetate kinase catalyzed phosphorylation of ADPβS, resulting in a mixture of (S_p/R_p)-ATPβS in a ratio of 0.56.[13] Attempts to use this approach for the synthesis (R_pS_p)-GTPαSβS at least in a mixture were not successful as (R_p)-GDPαSβS formed an instantaneous precipitate with this cation.

A mixture of all four diastereomers of GTPαSβS can be obtained by chemical synthesis (route B, Scheme I). Activation of GDPαSβS with diphenyl phosphorochloridate and reaction of the activated derivative with orthophosphate was not suitable because of the instability of GDPαSβS tri-n-butylammonium salt in DMF as reported previously for ADPβS.[14,15] However, the diastereomers of GTPαSβS could be prepared in a reaction involving 2′,3′-diacetylguanosine 5′-(α,β-dithiocyclotriphosphate) by a route that has recently been described for the synthesis of 3′-deoxy-3′-azidothymidine 5′-O-(1,3-dithiotriphosphate).[12] In this procedure 2 is reacted with thiopyrophosphate to give a mixture of GTPαSβS and GTPαSγS. The latter is selectively hydrolyzed to GDPαS, which can be removed by DEAE-Sephadex chromatography, and GTPαSβS can be isolated in good yield as a mixture of the four diastereomers as shown by the ^{31}P NMR spectrum (Figure 1). These diastereomers of GTPαSβS could be separated by HPLC (Scheme I, route B) into two groups, one containing (S_pS_p)- and (S_pR_p)-GTPαSβS (I), the other (R_pS_p)- and (R_pR_p)-GTPαSβS (II). The configurational assignments of these products are based on those of the GDPαSβS isomers. Thus, only one isomer of GDPαSβS is a substrate for phosphorylation with pyruvate kinase. This defines it as the α S_p isomer.[1] The product obtained from the enzymatic phosphorylation of (S_p)-GDPαSβS is identical by HPLC with the chemically synthesized GTPαSβS I isomer (Scheme I, route B), whereas chemically synthesized GTPαSβS II is identical with the acetate kinase product obtained from (R_p)-GDPαSβS.

When the mixture of (R_pS_p, R_pR_p)-GTPαSβS is hydrolyzed with glycerol kinase only (R_pR_p)-GTPαSβS reacts with the formation of (R_p)-GDPαSβS, leaving (R_pS_p)-

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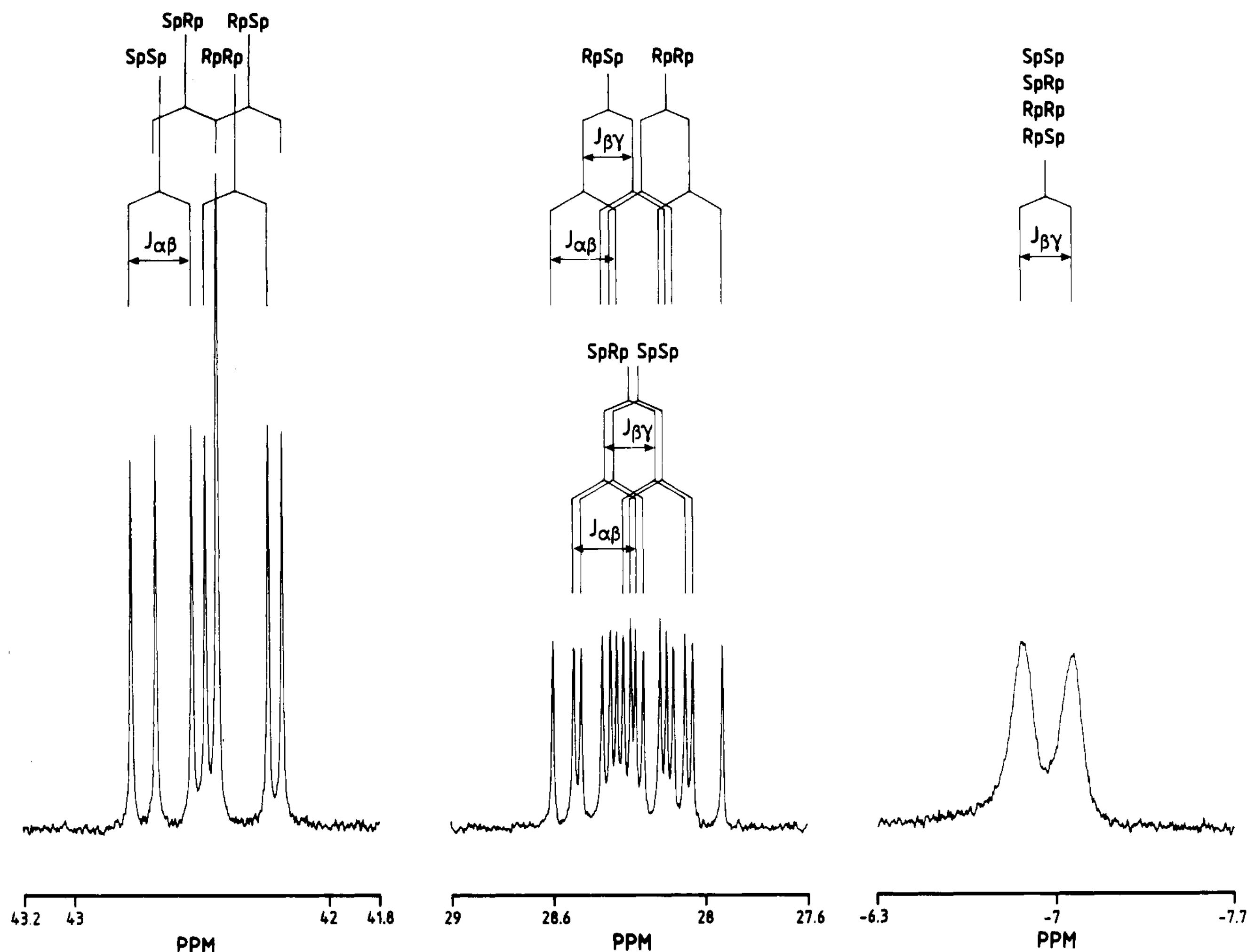

Figure 1. Selected regions of the ^{31}P NMR spectrum of the triethylammonium salts of a mixture of the four diastereomers of GTPαSβS. Peaks were assigned by comparison with mixtures of known amounts of pure diastereomers. Parameters were as follows: offset, 1600 Hz; sweep width, 15151 Hz; pulse width, 3.0 μs; 32K transients; acquisition time, 2.16 s; line broadening, 0.46 Hz; number of transients, 4000.

Table I. ^{31}P NMR Spectral Data[a]

	EDTA					Cd^{2+}				
	αP	βP	γP	$J_{P\alpha-P\beta}$	$J_{P\gamma-P\beta}$	αP	βP	γP	$J_{P\alpha-P\beta}$	$J_{P\gamma-P\beta}$
(S_pS_p)-GTPαSβS	42.64 (d)	28.26 (q)	−6.96 (d)	35.12	27.95	39.65 (d)	24–24.8 (q)	−9.40 (d)	29.32	20.85
(S_pR_p)-GTPαSβS	42.54 (d)	28.29 (q)	−6.96 (d)	35.26	27.95	39.63 (d)	24–24.8 (t)	−9.32 (d)	23.68	23.37
(R_pR_p)-GTPαSβS	42.35 (d)	28.15 (q)	−6.96 (d)	35.59	27.95	39.52 (d)	24–24.8 (q)	−9.47 (d)	29.17	20.62
(R_pS_p)-GTPαSβS	42.30 (d)	28.37 (q)	−6.96 (d)	36.80	27.95	39.34 (d)	24–24.8 (t)	−9.25 (d)	23.89	23.91

[a] Chemical shifts are in ppm. Coupling constants are in hertz.

GTPαSβS unchanged. This mixture can be separated by DEAE-Sephadex chromatography. The same treatment of the mixture of (S_pS_p)- and (S_pR_p)-GTPαSβS leads to hydrolysis of the latter, thus making (S_pS_p)-GTPαSβS available.

Myosine has the opposite selectivity for the configuration at the β-phosphorothioate[16] and was used for the synthesis of (R_pR_p)- and (S_pR_p)-GTPαSβS in similar reactions. Whereas the S_pR_p isomer could be obtained easily in pure form, the hydrolysis of the R_pS_p isomer was very slow and in this case only a 3:1 ratio of (R_p:S_p)-GTPαSβS could be obtained. This points to a problem inherent in route B, where starting material and product are not separable by DEAE-Sephadex chromatography. Thus, the stereochemical purity of the product depends on the quantitative digestion of the other isomer.

An interesting observation was made on oxidation of a mixture of the four diastereomers of GTPαSβS with I_2. The ^{31}P NMR spectrum of the reaction solution changed and showed signals at 17.91 ppm (d, Pα), 4.66 ppm (dd, Pβ), and −6.66 ppm with $J_{\alpha\beta}$ = 3.45 Hz and $J_{\beta\gamma}$ = 28.9 Hz. This spectrum is consistent with that of compound **6**, which arose by an intramolecular oxidation to form a disulfide bond and subsequent hydrolysis at either Pα or Pβ. This interpretation gains support from the spectrum obtained after addition of mercaptoethanol to the solution. The reaction mixture showed signals at 44.68 ppm (s), 34.56 ppm (d), and −9.21 ppm (d) J = 28.3 Hz in a 1:1 ratio. The signal at lowest field is due to GMPS, whereas those at 34.56 and −9.21 ppm are characteristic for thiopyrophosphate.

All products were characterized by reverse phase HPLC, UV, ^{31}P NMR (Table I), ^{13}C NMR (Table II), and PD mass spectroscopy. The UV spectra were identical with that of GTP. The ^{31}P NMR spectral data of the four diastereo-

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Table II. ^{13}C NMR Spectral Data[a]

	C-6	C-2	C-4	C-8	C-5	C-1′	C-4′ ($^3J_{P\alpha C4'}$)	C-3′	C-2′	C-5′ ($^2J_{P\alpha C5'}$)
GTP	162.50	158.96	155.78	141.80	118.67	92.23	88.48 (9.20)	78.73	74.68	69.69 (5.33)
GTPαSβS α(S_p) β(S_p&R_p)	163.35	158.37	156.21	142.41	120.57	91.32 91.28	88.38 (9.9)	78.57	75.14	69.96 (6.5) 69.92 (6.5)
GTPαSβS α(R_p) β(S_p&R_p)	163.35	158.37	156.18	142.23	120.57	91.21 (9.57)	88.34	78.57	75.07	70.35 (5.72)

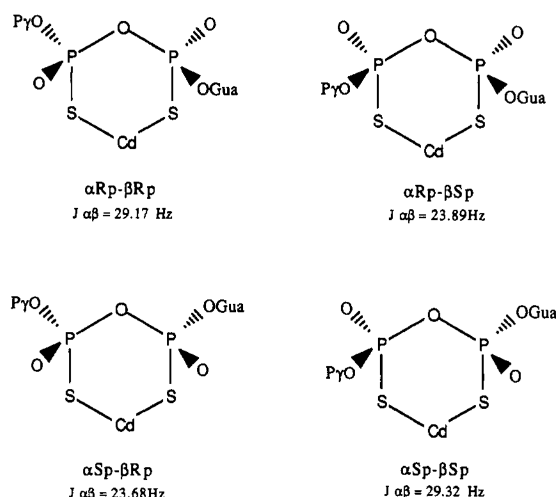
[a] Chemical shifts are in ppm. Coupling constants are in hertz.

mers are shown in Figure 1 and Table I. They permit the configurational assignment of all diastereomers. Since ^{31}P NMR spectroscopy also distinguishes between β-phosphorothioate diastereomers, this method can be used to monitor the enzymatic phosphorylations as an alternative to the earlier method of back digestions with enzymes of known specificity.[9]

Table I not only summarizes the NMR parameters obtained for the diastereomers of GTPαSβS in the presence of EDTA but also those in the presence of Cd^{2+}. The observed chemical shift changes of the α- and β-phosphorus for the Cd^{2+} complexes of **5** are similar to those reported for ATPαS and ATPβS.[3] Whereas in the spectra recorded in the presence of EDTA and also those with Mg^{2+} (not shown) the Pαβ and Pβγ coupling constants are nearly identical, the coupling constants (and the multiplicity of the Pβ signal) of the spectra of the Cd complexes are different for the S_pS_p, R_pR_p and the S_pR_p, R_pS_p diastereomer pairs. Coordination of the two sulfurs at Pα and Pβ can lead to the formation of six-membered rings, which would differ in the arrangement of the guanosine and the γ-phosphate at the Pα and Pβ, respectively. The position of these substituents will be determined by the configuration of the two phosphorothioate residues, giving rise to different arrangements for the S_pS_p, R_pR_p and S_pR_p, S_pR_p combinations (Scheme III). The S_pR_p and R_pS_p isomers are able to form a complex in which the guanosine and the γ-phosphate are on the same side of the six-membered ring, either above or below, whereas in that with the S_pS_p and R_pR_p isomers these residues will be on opposite sides. The Pαβ and Pβγ coupling constants are apparently determined by the arrangement of the two substituents in that they are nearly identical for the two members of each pair. Spectra reported earlier for the Cd complexes of the monophosphorothioates GTPαS and GTPβS had also not shown a difference in the Pαβ and Pβγ coupling constant.[17] The possibility of locking the diastereomers of GTPαSβS in six-membered rings of defined configuration should make them interesting for the determination of the configurational requirement of the substrate of individual members of the G-protein family.

^{13}C NMR spectra were recorded for the single diastereomers as well as for the S_pS_p/S_pR_p and R_pR_p/R_pS_p mixtures of the diastereomers of GTPαSβS. The latter was necessary to unambiguously identify small differences in shifts between the diastereomers. Peak assignment was based on literature data.[18] The signals for the purine carbons appear at somewhat lower field for the GTPαSβS than for GTP. They are, however, essentially identical for all diastereomers with the exception of C-8, whose signal is at lower field in the S_pS_p/S_pR_p than the R_pS_p/R_pS_p mixture. This is the first instance where a difference in chemical shift is observed in the base for diastereomers of nucleoside phosphorothioates. In keeping with the observation made with the diastereomers of 3′-deoxy-3′-azidothymidine 5′-O-(1-thiotriphosphate),[12] some significant differences in chemical shifts for most of the sugar carbons can be observed. This difference is most pronounced for C-1′ and C-5′. Interestingly, even the two diastereomers of (S_pS_p)- and (S_pR_p)-GTPαSβS show different chemical shifts for these two carbons.

Scheme III



Experimental Section

Materials and Methods. The phosphitylating agent 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one was purchased from Aldrich. Sublimed sulfur was from Merck Darmstadt and dried before use in a desiccator over P_2O_5. Dry dioxane and pyridine (containing less than 0.01% water) were also from Merck Darmstadt and were used as supplied. DMF and tri-*n*-butylamine were obtained from Fluka. All solvents were stored over 4-Å molecular sieves. 2′,3′-Diacetylguanosine was obtained from Bachem Biochemica (Heidelberg, FRG). Acetate kinase (*Escherichia coli*, suspension in ammonium sulfate, 1 mg/mL, 200 units/mg), pyruvate kinase (rabbit muscle, glycerol solution, 10 mg/mL, 200 units/mg), glycerol kinase (500 units/mL), acetyl phosphate (potassium–lithium salt), and phosphenol pyruvate (tricyclohexylammonium salt) were purchased from Boehringer Mannheim. Myosin was prepared as described[19] and stored as a suspension in 50% glycerol.

S-2-Cyanoethyl phosphorothioate was synthesized as described.[20] Trilithium phosphorothioate decahydrate was prepared according to Åkerfeldt.[21]

^{31}P NMR spectra were recorded at 145.78 Hz with broad-band decoupling. Samples were 2–4 mM of the nucleotide triethyl-

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ammonium salts in aqueous solution containing 20% D_2O and approximately 10 mM EDTA, pH 8 with 80% aqueous phosphoric acid as external standard. Spectra of the Cd^{2+} complexes were recorded with 2 mM GTPαSβS, 4 mM EDTA, and 20 mM $CdCl_2$. Spectra of the intermediates were recorded by addition of DMF-d_7 to the reaction solution. Chemical shifts are given in ppm and are positive when downfield from the standard. ^{13}C NMR spectra were recorded at 90.55 MHz with ^1H broad-band decoupling of 130 mM solutions of the sodium salts of the nucleotides in D_2O using the sodium salt of 3-(trimethylsilyl)propionic acid-d_4 as internal standard (δ 1.70 ppm). Plasma desorption mass spectra were recorded in the negative ion mode as previously described.[12]

Columns for analytical and preparative reverse-phase HPLC were packed with ODS Hypersil (5 μm, from Shandon Southern, Runcon, UK) and were eluted with 100 mM triethylammonium bicarbonate (TEAB), pH 7.5, containing a linear gradient of acetonitrile from 0% to 15% in 15 min. Chromatography on DEAE-Sephadex was carried out at 4 °C. Fractions containing product were combined and evaporated to dryness on a rotary evaporator and the residue was coevaporated with methanol to remove traces of buffer.

The tri-*n*-butylammonium salt of *S*-2-cyanoethyl phosphorothioate was prepared by adding a solution of *S*-β-cyanoethyl phosphorothioate disodium salt (1 mmol, 211 mg) in 10 mL of water onto a Merck cation exchange column (pyridinium form, 2 × 10 cm). The column was washed with 50 mL of water. The solution was evaporated, the residue was dissolved in 2 mL of methanol, tri-*n*-butylamine (1 mmol, 238 μL) was added, and the homogeneous solution evaporated.

Route A. Guanosine 5′-*O*-(1,2-Dithiodiphosphate) (GDPαSβS) (4). 2′,3′-Diacetylguanosine (367 mg, 1 mmol) was dissolved in pyridine (2 mL) and DMF (8 mL) and evaporated to dryness, and the residue was dried in vacuo overnight over P_2O_5 and the dissolved in the same solvents. To this solution was added under argon 1 mL of a 1 M solution of 2-chloro-4*H*-1,3,2-dioxaphosphorin-4-one in dioxane (1 mmol). After a 10-min reaction, 3 mL of a 1 M solution of the mono tri-*n*-butylammonium salt of *S*-2-cyanoethyl phosphorothioate in anhydrous DMF and tri-*n*-butylamine (1.42 mL, 6 mmol) was added. This was followed 10 min later by addition of a suspension of sulfur (128 mg, 4 mmol) in DMF (2 mL). (^{31}P NMR at this stage of the reaction showed the prescence of compound 3.) Water (10 mL) was added after 10 min and the reaction mixture evaporated to dryness 30 min later. The residual oil was dissolved in water (30 mL), and 1 N NaOH (30 mL) was added. The removal of the 2-cyanoethyl group was complete after 150 min, as monitored by TLC. The solution was put on a Dowex 50 × 2 column (2.5 × 30 cm) (pyridinium form), and the column was washed with water (200 mL). The effluent was concentrated to approximately 100 mL and the pH adjusted to 9.5 by addition of concd ammonia before application to a DEAE-Sephadex A-25 column (3 × 70 cm). The column was eluted with a linear gradient of 1250 mL each of 0.05 and 1.0 M TEAB. The diastereomers of GDPαSβS (4428 A_{254} units, 32%) were eluted as a mixture at the end of the gradient. NMR spectral data are summarized in Table I and II. The diastereomers were separated by preparative HPLC. HPLC retention times (min): 6.12 (S_p), 6.83 (R_p).

(R_pR_p)-Guanosine 5′-*O*-(1,2-Thiotriphosphate) ((R_pR_p)-GTPαSβS). A solution (total volume 10 mL) containing 80 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM acetyl phosphate, 2 mM dithiothreitol, 5.7 mM (R_p)-GDPαSβS, and acetate kinase (1000 units) was incubated at 37 °C. The progress of the reaction was followed by HPLC. The reaction was complete after incubation for 3 h. The reaction solution was chromatographed on a DEAE-Sephadex A-25 column (2 × 30 cm), which was eluted with a linear gradient of 1000 mL each of 0.5 and 1.0 M TEAB. The desired product was eluted between 0.72 and 0.73 M buffer. The product-containing fractions were combined and evaporated to dryness, and the residue was reevaporated with methanol to remove traces of buffer; yield of GTPαSβS, 325 A_{252} units (42%). NMR spectral data are summarized in Tables I and II. HPLC retention time (min): 7.53. Mass spectrum: M_r calcd 555.3, found for $(M - H)^-$, 553.8.

(S_pR_p)-Guanosine 5′-*O*-(1,2-Dithiotriphosphate) ((S_pR_p)-GTPαSβS). The phosphorylation reaction was performed exactly as described above for the synthesis of (R_pR_p)-GTPαSβS except that the reaction solution contained 5.7 mM of (S_p)-GDPαSβS. It was incubated at 37 °C for 1 h. The (S_pR_p)-GTPαSβS was isolated as described above in 38% yield (300 A_{252} units). NMR spectral data are summarized in Tables I and II. HPLC retention time (min): 6.83. Mass spectrum: M_r calcd 555.3, found for $(M - H)^-$, 554.5.

(S_pS_p)-Guanosine 5′-*O*-(1,2-Dithiotriphosphate) ((S_pS_p)-GTPαSβS). A solution (total volume 14.5 mL) containing 136 mM KCl, 55 mM Tris-Cl, pH 7.5, 1.5 mM dithiothreitol, 17 mM $MgCl_2$, a mixture of (S_p)- and (R_p)-GDPαSβS, 4.3 mM each, 35 mM phosphoenol pyruvate, and 1000 units of pyruvate kinase was incubated for 10 h at 37 °C. It was chromatographed on a DEAE-SEphadex A-25 column (3 × 60 cm) with a linear gradient of 1000 mL each of 0.5 and 1.0 M TEAB. The (S_pS_p)-GTPαSβS eluted between 0.70 and 0.72 M buffer and was isolated in 35% yield (22 μmol, 300 A_{252} units); HPLC retention time 6.83 min. It was contaminated with approximately 10% of the S_pR_p isomer as shown by ^{31}P NMR spectroscopy. This was removed by digestion with glycerol kinase. The reaction solution (total volume 1 mL) contained 100 mM glycine–KOH pH 9.4, 10 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM glycerol, 10 mM of the mixture of the two diastereomers of GTPαSβS, and 20 units of glycerol kinase. After incubation for 180 min at 37 °C the S_pR_p diastereomer had been completely hydrolyzed and the solution was again chromatographed as described above; Yield 90 A_{252} units (6.6 μmol). NMR spectral data are summarized in Tables I and II. Mass spectrum: M_r calcd 555.3, found for $(M - H)^-$, 554.5.

Route B. Guanosine 5′-*O*-(1,2-Dithiotriphosphate) (GTPαSβS). 2′,3′-Diacetylguanosine (36.7 mg, 0.1 mmol) was dissolved in pyridine (0.2 mL) and DMF (0.8 mL), the solution was evaporated to dryness, and the residue was dried in vacuo overnight over P_2O_5 and then dissolved in the same solvents. To this solution was added under argon 100 μL of a 1 M solution of 2-chloro-4*H*-1,3,2-dioxaphosphorin-4-one in dioxane (100 μmol). After 10 min, thiopyrophosphate bis(tri-*n*-butylammonium salt)[12] (200 μmol) in DMF (400 μL) was added together with tri-*n*-butylamine (95.2 μL, 400 μmol). After 10 min of stirring, a suspension of sulfur (6.4 mg, 200 μmol) in DMF (200 μL) was added, followed by water (2 mL) after an additional 10 min. After stirring for a further 30 min, the reaction mixture was evaporated to dryness, the residue was dissolved in water (15 mL), and 1.5 mL of 30% acetic acid was added. This solution was kept at 40 °C for 20 h in a closed flask. GTPαSγS was hydrolyzed quantitatively to GDPαS under these conditions (GTPαSβS/GDPαS ratio as determined by ^{31}P NMR was approximately 6:1).[12] This solution was evaporated, then redissolved in water, and applied to a DEAE-Sephadex column (2 × 40 cm), which was eluted with a linear gradient of 1000 mL each of 0.1 and 1.3 M TEAB. GTPαSβS was eluted between 0.88 and 0.95 M buffer. Product-containing fractions were pooled and evaporated to dryness, and the residue was coevaporated with methanol (4×) to remove traces of buffer; yield 592 A_{252} units (43 μmol, 43%). The same procedure could be used on 0.3-mmol scale with similar yield. The NMR spectrum of this product is shown in Figure 1. HPLC retention times (min): 6.83 (S_pR_p, S_pS_p) and 7.53 (R_pR_p, R_pS_p). The preparative HPLC separation into product groups R_pR_p, R_pS_p and S_pR_p, S_pS_p was performed under conditions as described in Materials and Methods.

(R_pS_p)-Guanosine 5′-*O*-(1,2-Dithiotriphosphate) (GTPαSβS). The reaction solution (total volume 2 mL) contained 100 mM glycine–KOH pH 9.4, 10 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM glycerol, 9.2 mM of the R_pR_p, R_pS_p mixture of GTPαSβS (252 A_{252} units), and 40 units of glycerol kinase. After incubation for 180 min at 37 °C, 50% of the starting material had been converted into (R_pR_p)-GDPαSβS (HPLC retention time 6.83 min). The reaction mixture was applied to a DEAE-Sephadex column (2 × 40 cm) and the elution was performed with a linear gradient of 0.1–1.0 M TEAB. (R_pS_p)-GTPαSβS was eluted between 0.95 and 0.98 M buffer; yield 94 A_{252} units (37%). HPLC retention time: 7.53 min. The isomeric purity of this material was determined by ^{31}P NMR spectroscopy (Table I).

(S_pR_p)-Guanosine 5′-*O*-(1,2-Dithiotriphosphate) (GTPαSβS). The reaction mixture (total volume 2 mL) contained 100 mmol Tris-Cl pH 7.4, 150 mmol $MgCl_2$, 2 mM dithiothreitol, 10 mmol of a mixture of (S_pR_p)- and (S_pS_p)-GTPαSβS, and 100

μL of a solution of myosin (10 mg/mL in 50% glycerol). The course of the reaction was followed by HPLC. After reaction for 60 and 120 min at 37 °C, a further 100 μL of myosin was added. After a 3-h incubation, 54% of the starting material had disappeared and a product with a HPLC retention time of 6.12 min had been formed. The reaction mixture was chromatographed as described above for the glycerol kinase reaction; yield of pure (S_pR_p)-GTPαSβS 125 A_{252} units (46%). The material was identical by ^{31}P NMR spectroscopy with that obtained by the enzymatic phosphorylation of (S_p)-GDPαSβS with acetate kinase.

(R_pR_p)-Guanosine 5'-*O*-(1,2-Dithiotriphosphate) (GTPαSβS). A mixture of (R_p,S_p)- and (R_pR_p)-GTPαSβS was reacted with myosine as described above. In this case the myosin-catalyzed hydrolysis was slow. Repeated additions of enzyme and longer reaction time (6 h) resulted only in a partial removal of the R_pS_p isomer. After DEAE-Sephadex purification, 8.7 μmol of a 3:1 mixture of (R_pR_p–R_pS_p)-GTPαSβS was obtained as analyzed by ^{31}P NMR spectroscopy.

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Tanzanene, a Spiro Benzopyranyl Sesquiterpene from *Uvaria tanzaniae* Verdc.

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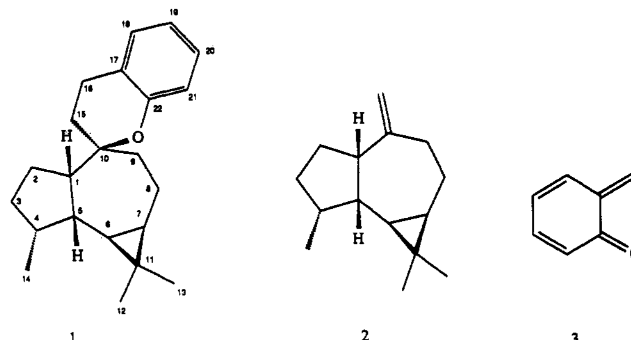
Tanzanene (1), a spiro benzopyranyl sesquiterpene, was isolated from the rootbark of *Uvaria tanzaniae* Verdc. and its structure was determined by high-resolution NMR. The molecular structure of tanzanene can be thought of as a cycloaddition product of alloaromadendrene (2) and the quinone methide of *o*-hydroxytoluene (6-methylene-2,4-cyclohexadien-1-one, 3). The known dihydrochalcones uvaretin, diuvaretin, chamuvaretin, and isotriuvaretin were also isolated from the same plant.

Introduction

As part of our search for new compounds with antimalarial activity from plants, we are investigating the genus *Uvaria*. The crude petroleum ether extract of the rootbark of *Uvaria tanzaniae* showed considerable activity against the multidrug resistant K_1 strain of *Plasmodium falciparum*[1] in vitro and was therefore studied in more detail. This led to the isolation of a new spiro benzopyranyl sesquiterpene, which we have named tanzanene (1), as well as the known dihydrochalcones uvaretin,[2] diuvaretin,[2] chamuvaretin,[2] and isotriuvaretin.[3] Tanzanene (1) has some features in common with other C-benzylated sesquiterpenes that have recently been isolated from *U. angolensis*[4] and *U. lucida* ssp. *lucida*.[5] However, unlike any other C-benzylated natural products reported so far, the C-benzyl substituent in 1 forms a spiro connection with the sesquiterpene part of the molecule.

Results and Discussion

Tanzanene (1) was isolated by silica gel chromatography of the petroleum ether extract of the rootbark of *Uvaria tanzaniae* using a gradient of hexane and ethyl acetate. The compound was obtained as white needles from methanol, mp 84–85 °C, $[\alpha]_D$ −4.5° (*c* 0.47, $CHCl_3$). The mass spectrum suggested an oxybenzylated sesquiterpene structure for 1, with characteristic fragments at *m/z* 310 (M^+, $C_{22}H_{30}O$), 203 ($[M - HOPhCH_2]^+$), 189 ($[M - HOPhCH_2CH_2]^+$), and 107 ($[HOPhCH_2]^+$). The UV (λ_{max} (EtOH) 284, 277, 225, 218 nm) and IR spectra (KBr, 1608, 1580 (C=C), 1247 (C—O), 746 cm^{-1} (ortho-substituted phenyl)) are in agreement with an *o*-oxybenzyl group in 1.[4,5]



The ^{1}H NMR spectrum of 1 indicated the presence of an alloaromadendrene (2) skeleton for the sesquiterpene

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