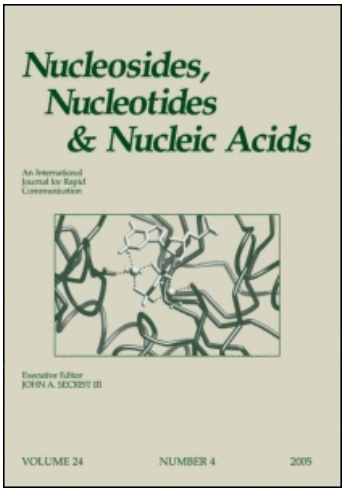

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NEW N-2-LABELLED FLUORESCENT DERIVATIVES OF GUANOSINE NUCLEOTIDES AND THEIR INTERACTION WITH GTP-BINDING PROTEINS

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ABSTRACT: Convenient syntheses of new fluorescent derivatives of guanosine nucleotides are described. The analogs bear a fluorescent group attached by an aliphatic linker containing 2 or 4 methylene groups on the N-2 position of the guanine base. They interact with the small GTPases H-Ras and cdc42 with an accompanying increase in fluorescence yield and display kinetic constants which are similar to those of unmodified nucleotides. The derivatives thus appear to be attractive alternatives to the widely used 2′(3′)-O-methylanthraniloyl derivatives and are likely to be useful for GTPases which do not tolerate modifications of the sugar moiety of the nucleoside.

Fluorescent nucleotide derivatives have proven to be of great value in delineating the mechanisms of nucleotide-utilizing systems, in particular those involving phosphate transfer from ATP or GTP. A class of derivatives which has been particularly useful are the methylanthraniloyl esters of the 2′- and 3′- hydroxyl groups of the nucleoside ribose moiety[1]. A series of studies with such derivatives of GTP, GDP and their phosphate-modified analogs has been crucial in all stages of the work leading to the detailed knowledge we now have of the mechanisms of action of GTPases, particularly of the Ras family[2-6]. These investigations are not limited to studies of the nucleotides with the respective GTPase, but are also critically important for examining the interactions of these proteins with others, including GAPs, exchange factors and effectors[7-9].

Despite many examples of useful applications, there are situations in which the ribose-modified derivatives do not bind to the protein of interest, and this has been particularly evident for ATP-dependent systems, for example the Hsp-70 class chaperone molecule DnaK, which will not tolerate modification of the ribose hydroxyls but will accept derivatives with a fluorescent group at the 8-position of the adenine moiety[10]. This approach did not appear to be promising for GTPases, since introduction of a bulky group at the 8-position of GTP led to a loss of a factor of ca. 1000 in affinity for H-Ras[5]. In contrast, a bulky aliphatic group at N-2 led only to slight weakening and a bulky aromatic group even to strengthening of the interaction[11, 12].

We were interested in increasing the diversity of analogs available for the investigation of GTPase mechanisms and the interaction of GTP-binding proteins with effectors and regulators for two main reasons. Firstly, some GTPases do not tolerate modification of the ribose hydroxyls. Secondly, we were interested in developing a chemical route which would lead to introduction of a reactive group which could be labelled with a variety of reagents, including fluorescent derivatives and potential affinity labels, without the necessity of repeating the whole synthesis for each derivative.

RESULTS AND DISCUSSION

Preparation of fluorescent N-2-amino-linker guanosine and GTP derivatives

It has been known for some time that GTP derivatives in which the N-2 group of the base is modified are accepted by GTP-binding proteins, including the Ras family, as long as one amine proton is still available for hydrogen bonding to Asp-119 in Ras or its equivalent in other proteins. We therefore looked for a route towards introduction of a linker moiety bearing a reactive amino group at this position. The synthetic approach adopted by Noonan et al.[11] could be used, but has the disadvantage that the difficult synthetic step of formation of the glycosidic linkage between the modified base and ribose has to be performed for each analog synthesized. The approach chosen in this work is outlined in Fig. 1 and starts with a natural product which is commercially available in large quantities, 5-amino-1-ß-D-ribofuranosyl-4-imidazolcarboxamid (AICA). As described by Yamazaki et al.[13] , AICA (I) reacts with sodium xanthogenate under basic conditions to give 2-mercaptoinosine (II). The mercapto group can then be

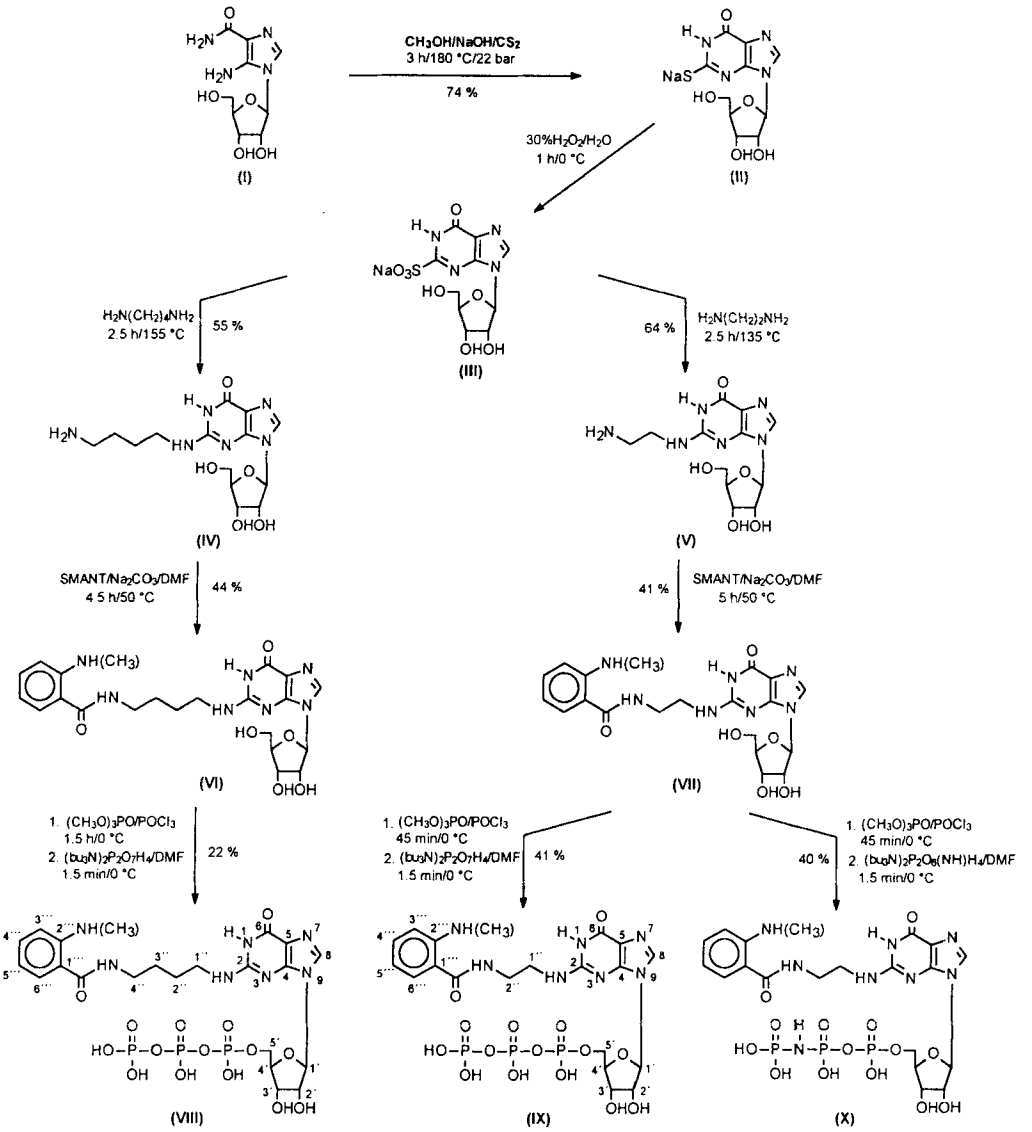

FIG. 1: Reaction scheme of the synthesis of the new N2-labelled fluorescent guanosine nucleotides N-2-(4′′-N-methylanthraniloylaminobutyl)guanosine-triphosphate (VIII), N-2-(2′′-N-methylanthraniloylaminoethyl)guanosine-triphosphate (IX) and N-2-(2′′-N-methylanthraniloylaminoethyl)guanosine-(β,γ-imido)triphosphate (X).

oxidized by hydrogen peroxide to the sulfonate derivative (III) (The synthesis of the stereoisomer 9-α-D-xylofuranosylinosin-2-sulfonate by the same method has been described earlier using 1-α-D-xylofuranosyl-4-carbamoyl-5-aminoimidazole as starting compound[14]). Without isolation of this intermediate, a reaction with any primary amine can be carried out. In Fig. 1, this is shown for two different diamines, namely diaminobutane and diaminoethane, to give the guanosine derivatives N-2-(4"-aminobutyl)guanosine (IV) and N-2-(2"-aminoethyl)guanosine (V). The large excess of amine used ensures that one molecule of linker does not react with two nucleoside molecules.

In the present work, the amino-linker guanosine derivatives IV and V were labelled with a fluorescent group at this stage of the synthesis. Succinimidyl-N-methylanthranilate (SMANT) was allowed to react with IV or V without prior protection of the sugar hydroxyl groups to give the fluorescent derivatives N-2-(4"-N-methylanthraniloylaminobutyl)guanosine (VI) and N-2-(2"-N-methylanthraniloylaminoethyl)guanosine (VII). These derivatives were converted to their triphosphates (VIII, mabGTP and IX, maeGTP) in a two-step one-flask reaction in a similar manner to that described by Ludwig[15]. In the case of the aminoethyl derivative, the non-hydrolyzable analog maeGppNHp (X) was also prepared.

Interaction of fluorescent N-2-aminolinker guanosine derivatives with GTPases

The effect of interaction of mabGTP and maeGTP with nucleotide-free H-Ras on the fluorescence properties of the nucleotides is shown in Fig. 2. It can be seen that there is a significant increase in fluorescence emission intensity in both cases, with a more pronounced effect in the case of maeGTP than for mabGTP. Interaction with cdc42 leads to a reversal of these comparative effects, with mabGTP showing a much larger change (95%) than maeGTP (25%) (data not shown). All interactions lead to a blue shift of 3-6 nm in the position of the emission maximum. Although we can only speculate on the reasons for these differences, it is possible that the longer linker in mabGTP allows the fluorescent group to interact with the 12 amino acids found as an insertion in proteins of the Rho family compared with Ras, this insertion occurring between beta sheet strand ß5 and alpha helix H4[16, 17].

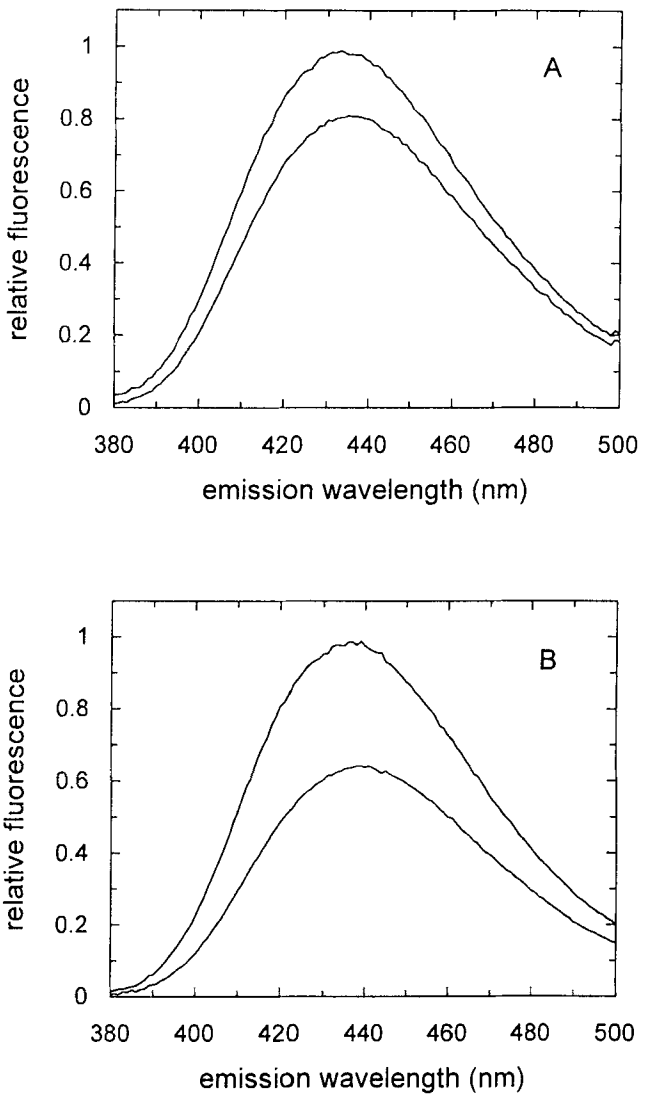

FIG. 2: Fluorescence emission spectra of A 100 μM mabGTP (lower curve) and after addition of 10 μM nucleotide-free H-Ras (upper curve) and B 100 μM maeGTP (lower curve) and after addition of 10 μM nucleotide-free H-Ras (upper curve). Measurements were performed in standard buffer at 20 °C as described in material and methods.

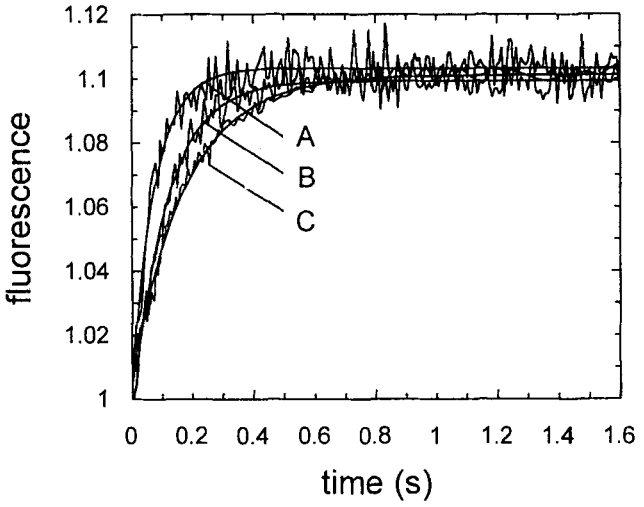

FIG. 3: Association kinetics of 10 μM mabGTP (A), maeGTP (B) and maeGppNHp (C) and 1.5 μM nucleotide-free Ras (A) and 1 μM nucleotide-free Ras (B and C), respectively, at 20 °C in standard buffer. Formation of the complex was monitored by measuring the increase of fluorescence of the methylanthraniloyl-group at N-2 of the nucleotides in a stopped-flow apparatus as described in material and methods. Data were fitted using a single exponential equation.

The fluorescence signals identified in the experiments of Fig. 2 can be used for kinetic studies of nucleotide association and dissociation. Examples for the time dependence of the signal change seen after rapid mixing of Ras with mabGTP, maeGTP and maeGppNHp are shown in Fig. 3. At the concentrations used (10 μM nucleotide and 1-1.5 μM protein), all transients could be well fitted using a single exponential equation. The observed pseudo-first order rate constant was determined as a function of nucleotide concentration, and the dependence on the concentration is shown in Fig. 4. As seen previously for the Ras interaction with nucleotides bearing methylanthraniloyl groups on the sugar hydroxyls[2] and also for the interaction of unlabelled nucleotides with a fluorescent Ras mutant (Y32W)[5], there is a hyperbolic dependence of the observed rate constant on the nucleotide concentration, suggesting a two step binding reaction. For maeGppNHp, the dissociation rate constant can also be measured by displacement by a non-labelled nucleotide (Fig. 5). For the triphosphate analogs, dissociation takes place on approximately the same time scale as the GTPase reaction, making its determination

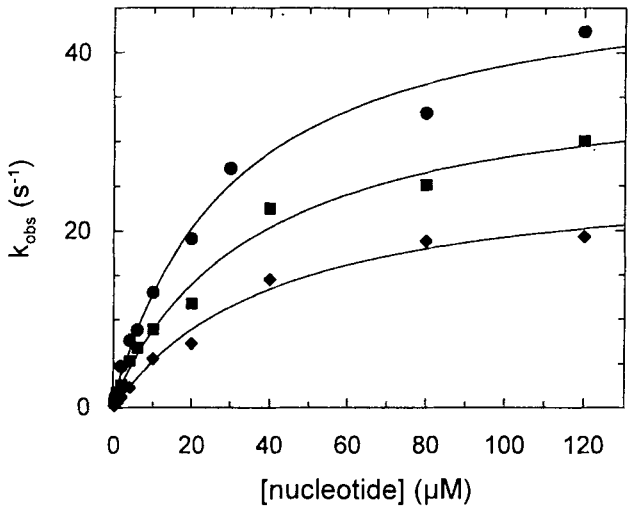

FIG. 4: Dependence of the pseudo-first order rate constants of the association of mabGTP (•), maeGTP (▪) and maeGppNHp (◆) and nucleotide-free Ras as a function of the nucleotide concentrations. Conditions are the same as in Figure 3. Fits to the data correspond to hyperbolic expressions.

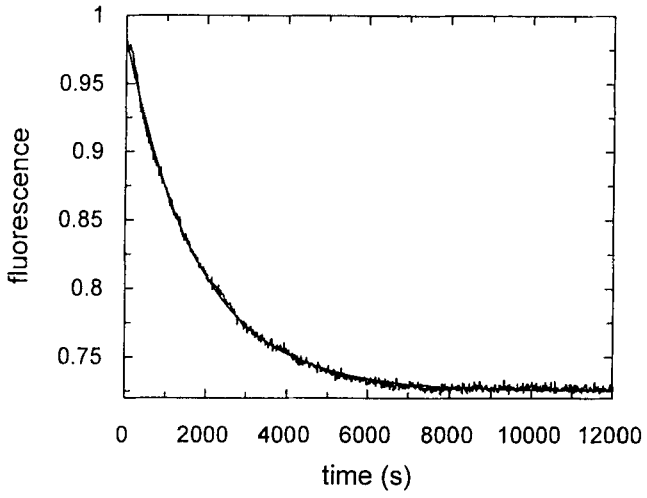

FIG. 5: Time course of the dissociation of maeGppNHp from a stoichiometric complex with Ras (50 nM). The displacement was performed in the presence of 50 μM non-labelled GppNHp at 20 °C in standard buffer as described in material and methods. The obtained data were fitted using a single exponential equation.

TABLE 1: Association and dissociation kinetics of mabGTP, maeGTP and maeGppNHp to Ras and the comparison to earlier results of the sugar methylanthraniloyl derivatives and the natural nucleotides

	$K_1 = k_1/k_{-1}$ (10^4 M^{-1})	k_2 (s^{-1})	$k_{on} = K_1 * k_2$ (10^6 $M^{-1}s^{-1}$)	$k_{off} = k_{-2}$ (10^{-4} s^{-1})	$K_A = k_{on}/k_{off}$ (10^9 M^{-1})
mabGTP	3.3	49.3	1.6		
maeGTP	2.7	37.5	1.0		
maeGppNHp	2.5	26.9	0.7	6.2	1.1
2′(3′)-mantGTP[22]	10.2	37.8	3.8	0.2	160
2′(3′)-mantGppNHp[23]	10.1	17.4	1.8	3.6	5
GTP[5]	12.5	21.8	2.7	0.3	159

Scheme for a two step binding mechanism:

$$\text{H-Ras} + \text{nucleotide} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{H-Ras}\bullet\text{nucleotide} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \text{H-Ras}^*\bullet\text{nucleotide}$$

more complex[18], so that this was not examined in the course of the present study. The values obtained for the kinetic parameters describing the Ras interaction with the new derivatives used in the present study are given in Table 1. They are compared with values obtained earlier for sugar methylanthraniloyl derivatives and the natural nucleotides. It can be seen that there is a tendency for the initial binding affinity to be somewhat weaker than for the natural or sugar modified derivatives, but that the second step occurs at a similar rate. Comparison of the dissociation rate constant of maeGppNHp and mantGppNHp shows that this is increased by a factor of ca. 2 with the new derivative, and taken together with the reduction in affinity in the first step, this leads to a decrease of a factor of ca. 5 in the overall affinity. However, this still has a value of 10^9 M^{-1}, which is high enough to allow formation of a stoichiometric complex between the protein and the nucleotide, which is an important prerequisite for certain types of experiments. Assuming that the same factor in the dissociation rates applies when comparing mabGTP or maeGTP with GTP, the overall affinity of the new analogs in their triphosphate form is estimated to be greater than 10^{10} M^{-1}.

The new GTP analogs prepared in this work were tested for their ability to be hydrolyzed by Ras. Fig. 6 shows the results of incubating a stoichiometric complex of

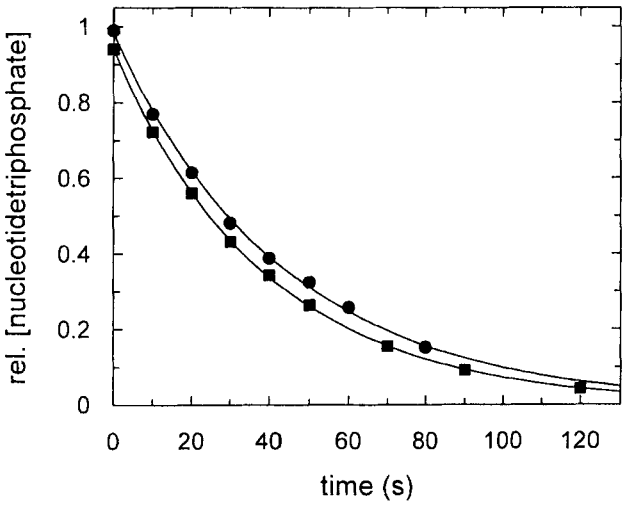

FIG. 6: Hydrolysis of mabGTP (•) and maeGTP (▪), assayed by incubating a stoichiometric complex of the corresponding nucleotide and Ras (150 μM) at 37 °C in standard buffer. The curves show the relationship of nucleoside-triphosphate to total nucleotide concentration as a function of time. The measurements were performed as described in material and methods. The data were fitted using a single exponential equation.

Ras.mabGTP or Ras.maeGTP in the presence of magnesium ions at 37 °C. The rate of disappearence of the triphosphates was monitored by reversed phase HPLC, and it can be seen that there was a single exponential decay occurring at similar rates in both cases (for mabGTP 0.023 min^{-1} and for maeGTP 0.026 min^{-1}). These rates are compared with those of GTP (0.028 min^{-1})[19] and mantGTP (0.025 min^{-1})[4] from earlier work, and it can be seen that they are essentially unaltered by the new modification. Thus, the large fluorescent residue on the exocyclic amino group of the guanine base does not interfere with the basal GTPase mechanism of Ras.

CONCLUSION

The chosen synthetic route to N-2 labelled fluorescent GTP analogs was successful and in its present form fulfils the requirement of giving the possibility of varying the labelling group without repeating the whole synthetic procedure. However, with the present scheme, the nucleoside phosphorylation procedure has to be performed in each

case. This situation could be improved in future work by going through the complete synthesis to give an appropriately amino-protected N-2 linker GTP. After removal of the protecting group, the reactive amino group could then be used for attaching fluorescent moieties or groups which could be used in affinity labelling studies (e.g. photoactivatable groups) or for attaching GTP or GDP to a polymer matrix for affinity chromatography.

The two new GTP derivatives synthesized (mabGTP and maeGTP) interact with Ras and cdc42 in a manner resembling that of the natural nucleotides or their fluorescent sugar-modified derivatives (mantGTP). As already shown for the mant nucleotides and for GTP and GDP, the association reaction occurs in two steps. In the case of mabGTP and maeGTP, the initial weak binding interaction is somewhat weakened, but the subsequent isomerization step and the hydrolysis reaction are essentially identical to the natural nucleotides, so that we can conclude that all of the important basic properties of GTP in terms of its interaction with Ras are retained after introduction of the fluorescent group at N-2. Thus, the analogs are likely to be of general use for investigations on GTP-binding proteins and could be particularly useful in cases where modifications of the sugar moiety are not tolerated or desired or when useful signals are not seen with the sugar modified derivatives.

EXPERIMENTALS

NMR-spectroscopy and electrospray-masspectrometry

^{1}H-NMR (500 MHz) and ^{13}C-NMR (125.7 MHz) spectra were recorded on a Bruker ADVANCE DRX-500 spectrometer in D_2O. Chemical shifts are relative to TSP as an internal standard. Electrospray-masspectra were recorded on a Finningan MAT 95 sectorfield-masspectrometer at the Institut für Spektrochemie und angewandte Spektroskopie in Dortmund.

Synthesis of the sodium salt of 2-mercaptoinosine (II)

10.0 g (38.6 mmol) 5-amino-1-β-D-ribofuranosyl-4-imidazole-carboxamide (AICA, Sigma) (I) and 11.6 ml (193 mmol) carbondisulfide were added to a solution containing 7.72 g (193 mmol) sodiumhydroxide in 70 ml methanol at 35 °C. The reaction mixture was stirred for 3 h at 180 °C in an autoclave (22 bar). After cooling to 0 °C in an

ice bath, the precipitate was collected by filtration and washed with 10 ml cold methanol. The residue was recrystallized twice from 30 ml water and dried under vacuum over P_2O_5 to yield 9.46 g (74 %) II (R_f = 0.73 (silica gel/isopropanol : water : ammonia 7 : 3 : 1)).

^{1}H-NMR (D_2O, 500 MHz): δ = 3.68 (dd, $J_{H\text{-}5'a\text{-}H\text{-}4'}$ = 3.43 Hz, $^1J_{H\text{-}5'a\text{-}H\text{-}5'b}$ = 12.96 Hz; 1H, H-5'a), 3.79 (dd, $J_{H\text{-}5'b\text{-}H\text{-}4'}$ = 2.56 Hz, $^1J_{H\text{-}5'b\text{-}H\text{-}5'a}$ = 12.96 Hz; 1H, H-5'b), 4.15 (m_c; 1H, H-4'), 4.30 (dd, $J_{H\text{-}3'\text{-}H\text{-}2'}$ = 4.99 Hz, $J_{H\text{-}3'\text{-}H\text{-}4'}$ = 3.27 Hz; 1H, H-3'), 4.66 (m ; 1H, H-2'), 5.80 (d, $J_{H\text{-}1'\text{-}H\text{-}2'}$ = 6.19 Hz; 1H, H-1'), 7.86 (s; 1H, H-8).

^{13}C-NMR (D_2O, 125.7 MHz): δ = 66.10 (t; C-5'), 75.29 (d; C-3'), 78.08 (d; C-2'), 90.48 (d; C-4'), 92.83 (d; C-1'), 124.17 (s; C-5), 143.05 (d; C-8), 154.93 (s; C-4), 163.99 (s; C-2), 165.23 (s; C-6).

N-2-(4''-aminobutyl)guanosine (IV)

1.00 g (3.02 mmol) mercaptoinosine (II) was dissolved in 11 ml water and cooled to 0 °C. After addition of 0.92 ml (9.00 mmol) 30 % hydrogen peroxide within 5 min, the mixture was stirred an additional hour at 0 °C. During the addition of hydrogen peroxide the temperature must be kept below 5 °C, because otherwise increased formation of a by-product, xanthosine, is observed. Subsequently 16.0 ml (160 mmol) 1,4-diaminobutane were added and the mixture was refluxed for 2.5 h (155 °C). Excess of 1,4-diaminobutane was removed under vacuum (41 °C/10^{-2} mbar) and the yellow oily residue was resolved in 60 ml water. For purification, the solution was applied to the cation-exchange resin Dowex 50 W x 4 100-200 mesh. After isocratic elution with 600 ml water the prodruct was eluted with 1 M NH_3. The effluent was monitored by measuring UV absorbance at 260 nm. The fractions estimated to contain pure N-2-(4''-aminobutyl)guanosine (IV) were pooled and lyophilized three times from water to yield 589 mg (55 %) IV (R_f = 0.31 (silica gel/isopropanol : water : ammonia 7 : 3 : 1)).

^{1}H-NMR (D_2O, 500 MHz): δ = 1.55-1.65 (m; 4H, H-2", H-3"), 2.91 (t, $J_{H\text{-}4''\text{-}H\text{-}3''}$ = 7.22 Hz; 2H, H-4"), 3.28 (t, $J_{H\text{-}1''\text{-}H\text{-}2''}$ = 6.53 Hz; 2H, H-1"), 3.69 (dd, $J_{H\text{-}5'a\text{-}H\text{-}4'}$ = 4.47 Hz, $^1J_{H\text{-}5'a\text{-}H\text{-}5'b}$ = 12.64 Hz; 1H, H-5'a), 3.77 (dd, $J_{H\text{-}5'b\text{-}H\text{-}4'}$ = 3.27 Hz, $^1J_{H\text{-}5'b\text{-}H\text{-}5'a}$ = 12.64 Hz; 1H, H-5'b), 4.10 (m_c; 1H, H-4'), 4.33 (dd, $J_{H\text{-}3'\text{-}H\text{-}2'}$ = 5.16 Hz, $J_{H\text{-}3'\text{-}H\text{-}4'}$ = 4.12 Hz; 1H, H-3'), 4.73 (m; 1H, H-2'), 5.82 (d, $J_{H\text{-}1'\text{-}H\text{-}2'}$ = 6.19 Hz; 1H, H-1'), 7.79 (s; 1H, H-8).

^{13}C-NMR (D_2O, 125.7 MHz): δ = 29.08, 30.15 (t; C-2", C-3"), 43.85, 44.98 (t; C-1", C-4"), 66.10 (t; C-5'), 75.07 (d; C-3'), 77.54 (d; C-2'), 89.47 (d; C-4'), 92.69 (d; C-1'), 121.22 (s; C-5), 142.15 (d; C-8), 156.10 (s; C-4), 159.74 (s; C-2), 166.30 (s; C-6).

N-2-(2´´-aminoethyl)guanosine (V)

2.00 g (3.04 mmol) mercaptoinosine (II) were oxidized to the inosine-2-sulfonate (III) in the same manner as described for IV. The substitution reaction was performed with a 100fold excess of 1,2-diaminoethane instead of a 50fold excess 1,4-diaminobutane as described for IV to yield 1.26 g (64 %) V (R_f = 0.35 (silica gel/isopropanol : water : ammonia 7 : 3 : 1)).

^{1}H-NMR (D_2O, 500 MHz): δ = 2.99 (t, $J_{H-2"-H-1"}$ = 5.50 Hz; 2H, H-2"), 3.33 (t, $J_{H-1"-H-2"}$ = 5.50 Hz; 2H, H-1"), 3.60 (dd, $J_{H-5'a-H-4'}$ = 4.47 Hz, $^1J_{H-5'a-H-5'b}$ = 12.72 Hz; 1H, H-5'a), 3.68 (dd, $J_{H-5'b-H-4'}$ = 3.10 Hz, $^1J_{H-5'b-H-5'a}$ = 12.72 Hz; 1H, H-5'b), 4.00 (m_c; 1H, H-4'), 4.21 (m_c; 1H, H-3'), 4.56 (t (dd), $J_{H-2'-H-3'}$ = 5.50 Hz, $J_{H-2'-H-1'}$ = 5.49 Hz; 1H, H-2'), 5.65 (d, $J_{H-1'-H-2'}$ = 5.49 Hz; 1H, H-1'), 7.63 (s; 1H, H-8).

^{13}C-NMR (D_2O, 125.7 MHz): δ = 44.20, 44.57 (t; C-1", C-2"), 65.99 (t; C-5'), 75.04 (d; C-3'), 77.87 (d; C-2'), 89.14 (d; C-4'), 93.17 (d; C-1'), 121.31 (s; C-5), 141.40 (d; C-8), 155.60, 160.74 (s; C-2, C-4), 167.14 (s; C-6).

N-2-(4´´-N´-methylanthraniloylaminobutyl)guanosine (VI)

250 mg (705 µmol) N-2-(4´´-aminobutyl)guanosine (IV) were dissolved in 14 ml of 0.5 M sodiumcarbonate. After addition of a 0.2 mM solution of 500 mg (2.01 mmol) succinimidyl-N-methylanthranylate (SMANT, Molecular Probes) in 10.6 ml dimethyl formamide (DMF), the mixture was vigorously stirred at 50 °C for 4.5 h. To quench the reaction, 15 ml concentrated ammonia were added. After additional stirring for 30 min at 50 °C the reaction mixture was diluted with 40 ml of water at 4 °C. The solvent was evaporated and the residue was dissolved in 15 ml water, microfiltrated and purified step-by-step by semi-preparative reversed-phase HPLC (Beckmann system Gold) (ODS-Hypersil, C_{18}-column, 5 µm, 120 Å, 240 x 8 mm; UV absorbance: 254 nm and 365 nm; flow rate: 2.5 ml/min; gradient: 0 % - 25 % buffer B during 15 min, 25 % buffer B isocratic for 10 min and 25 % - 60 % buffer B during 15 min (buffer A: 100 mM

triethylammoniumacetate pH 7.0, buffer B: 70 % acetonitrile)). The elution of the nucleoside was observed after 35 min. Pools containing the nucleoside were combined and the solvent was briefly evaporated on a rotary evaporator. To completely remove triethylamine, the residue was lyophilized three times from 5 ml water to yield 150 mg (44 %) of the fluorescent derivative VI (R_f = 0.59 (reversed-phase/methanol : water : acetonitrile 6 : 4 : 1)).

^{1}H-NMR (D_2O, 500 MHz): δ = 1.57-1.68 (m; 4H, H-2'', H-3''), 2.64 (s; 3H, CH_3), 3.26-3.33 (m; 4H, H-1'', H-4''), 3.66 (dd, $J_{H\text{-}5'a\text{-}H\text{-}4'}$ = 4.82 Hz, $^1J_{H\text{-}5'a\text{-}H\text{-}5'b}$ = 12.46 Hz; 1H, H-5'a), 3.75 (dd, $J_{H\text{-}5'b\text{-}H\text{-}4'}$ = 2.92 Hz, $^1J_{H\text{-}5'b\text{-}H\text{-}5'a}$ = 12.46 Hz; 1H,H-5'b), 4.02 (m_c; 1H, H-4'), 4.28 (m_c; 1H, H-3'), 4.59 (m_c; 1H, H-2'), 5.72 (d, $J_{H\text{-}1'\text{-}H\text{-}2'}$ = 4.81 Hz; 1H, H-1'), ABCD-Signal (δ_A = 6.44, δ_B = 6.65, δ_C = 7.11, δ_D = 7.22, $J_{A\text{-}C}$ = 7.21 Hz, $J_{A\text{-}D}$ = 7.90 Hz, $J_{B\text{-}D}$ = 8.25 Hz; 4H, H-5''', H-3''', H-6''', H-4'''), 7.77 (s; 1H, H-8).

^{13}C-NMR (D_2O, 125.7 MHz): δ = 26.49, 26.70 (t; C-2'', C-3''), 29.44 (q; CH_3), 38.68, 40.36 (t; C-1'', C-4''), 61.78 (t; C-5'), 70.68 (d; C-3'), 73.58 (d; C-2'), 85.40 (d; C-4'), 87.05 (d; C-1'), 110.57 (d; C-3'''), 114.06 (d; C-5'''), 115.44 (s; C-5), 117.04 (s; C-1'''), 128.32 (d; C-6'''), 132.35 (d; C-4'''), 136.23 (d; C-8), 150.90, 151.10, 152.86 (s; C-2, C-4, C-2'''), 156.90 (s; Ar-CO), 169.23 (s; C-6).

N-2-(2''-N'-methylanthraniloylaminoethyl)guanosine (VII)

329 mg (1.01 mmol) N-2-(2''-aminoethyl)guanosine (V) were labelled with the fluorophore, SMANT, at the terminal amino group at N-2 of the purine base in the same manner as described for VI. The step-by-step purification by semi-preparative reversed-phase HPLC was performed as described for VI, with a modified gradient: 0 % - 22.5 % buffer B during 5 min, 22.5 % buffer B isocratic for 10 min, 22.5 % - 43 % buffer B during 5 min, 43 % buffer B isocratic for 6 min. The elution of the nucleoside was observed after 21 min. The yield was 192 mg (41 %) fluorescent derivative VII (R_f = 0.61 (reversed-phase/methanol : water : acetonitrile 6 : 4 : 1)).

^{1}H-NMR (D_2O, 500 MHz): δ = 2.58 (s; 3H, CH_3), 3.42-3.61 (m; 4H, H-1'', H-2''), 3.64 (dd, $J_{H\text{-}5'a\text{-}H\text{-}4'}$ = 4.64 Hz, $^1J_{H\text{-}5'a\text{-}H\text{-}5'b}$ = 12.54 Hz; 1H, H-5'a), 3.74 (dd, $J_{H\text{-}5'b\text{-}H\text{-}4'}$ = 3.27 Hz, $^1J_{H\text{-}5'b\text{-}H\text{-}5'a}$ = 12.54 Hz; 1H, H-5'b), 3.98 (m_c; 1H, H-4'), 4.20 (m_c; 1H, H-3'), 4.33 (m_c; 1H, H-2'), 5.61 (d, $J_{H\text{-}1'\text{-}H\text{-}2'}$ = 4.12 Hz; 1H, H-1'), ABCD-Signal (δ_A = 6.47,

δ_B = 6.60, δ_C = 7.05, δ_D = 7.72, J_{A-C} = 7.73 Hz, J_{A-D} = 7.82 Hz, J_{B-D} = 8.25 Hz, $^4J_{C-D}$ = 1.55 Hz; 4H, H-5''', H-3''', H-6''', H-4'''), 7.76 (s; 1H, H-8).

^{13}C-NMR (D_2O, 125.7 MHz): δ = 30.20 (q; CH_3), 40.21, 42.14 (t; C-1'', C-2''), 63.16 (t; C-5'), 72.10 (d; C-3'), 75.93 (d; C-2'), 86.82 (d; C-4'), 90.16 (d; C-1'), 112.26 (d; C-3'''), 116.18 (d; C-5'''), 116.66, 117.22 (s; C-5, C-1'''), 129.44 (d; C-6'''), 134.07 (d; C-4'''), 138.61 (d; C-8), 151.75, 152.94, 154.59 (s; C-2, C-4,C-2'''), 159.80 (s; Ar-CO), 173.03 (s; C-6).

N-2-(4''-N'-methylanthraniloylaminobutyl)guanosine-5'-triphosphate (VIII)

65 mg (0.13 mmol) N-2-(4''-N'-methylanthraniloylaminobutyl)guanosine (VI) (dried over P_2O_5) were dissolved (under dry argon) in 0.5 ml dry trimethylphosphate at 40 °C. After cooling to 0 °C, 48 µl (0.52 mmol) phosphorus oxychloride ($POCl_3$) were added. The reaction was monitored by analytical reversed-phase HPLC (ODS-Hypersil, C_{18}-column, 5 µm, 120 Å, 250 x 4.6 mm; UV absorbance: 254 nm; flow rate: 1.5 ml/min; gradient: 15 % buffer B isocratic for 1 min, 15 % - 100 % buffer B during 14 min; buffer A: 10 mM tetrabutylammoniumbromide, 100 mM potassium phosphate buffer (pH 6.5); buffer B: 50 % acetonitrile and 50 % buffer A; retention times of the nucleoside (mabG), nucleosidemonophosphate (mabGMP), nucleosidediphosphate (mabGDP) and nucleosidetriphosphate (mabGTP) were 10.2 min, 11.3 min, 11.7 min and 12.0 min respectively). After stirring for 1.5 h at 0 °C, the excess of phosphorus oxychloride was removed under vacuum (10 min). Subsequently a solution of 520 mg (1.14 mmol) tributylammoniumpyrophosphate (1.5 mol tributylphosphate/mol pyrophosphoric acid) and 610 µl (2.56 mmol) tributylamine in 2 ml DMF was added to the phosphorodichloridate at 0 °C. The reaction was stopped after 1.5 min by addition of 50 ml 0.2 M triethylammoniumhydrogencarbonate ($TEAHCO_3$) (pH 7.5). The buffer was removed under vacuum and the residue dissolved in 50 ml water. After adjusting the pH to 7.5 with 1 M sodiumhydroxide, the solution was extracted with 40 ml ether. For further purification the solution was applied to the quarternary anion-exchange resign Q-Sepharose (100 x 15 mm; Pharmacia). After isocratic elution with 200 ml 50 mM $TEAHCO_3$, the product was eluted with a linear gradient from 50 to 500 mM $TEAHCO_3$ (pH 7.5). The effluent was monitored by measuring UV absorbance at 254 nm; the

mabGTP was eluted between 400 and 470 mM $TEAHCO_3$. The fractions estimated to contain pure mabGTP were pooled and lyophilized five times from 5 ml water.

The final concentration of mabGTP was determined by UV absorbance with $\varepsilon_{252} = 22.600$ at pH 7.5 to yield 19.8 mg (22 %) VIII; ES-MS: m/z = 726 $(M-H)^-$.

Enzymatic dephosphorylation with alkaline phosphatase yields mabGDP, mabGMP and finally the nucleoside VI. The process was monitored by reversed-phase HPLC as desribed above.

N-2-(2′′-N′-methylanthraniloylaminoethyl)guanosine-5′-triphosphate (IX)

75 mg (0.16 mmol) N-2-(2′′-N′-methylanthraniloylaminoethyl)guanosine (VII) were phosphorylated in a similar manner to that described for VIII. The activation of the nucleoside occured with a 7 molar excess of $POCl_3$ instead of a 4 molar excess as described for mabGTP. After 45 min 87 % of the monophosphorodichloridate were formed and excess of $POCl_3$ was removed under vacuum. The phosphorylation step was performed as described for VIII. The retention times of the nucleoside (maeG), nucleosidemonophosphate (maeGMP), nucleosidediphosphate (maeGDP) and nucleosidetriphosphate (maeGTP) were 8.8 min, 10.0 min, 10.8 min and 11.2 min respectively. The yield of maeGTP was 46 mg (41 %); ES-MS: m/z = 698 $(M-H)^-$.

Enzymatic dephosphorylation with alkaline phosphatase yields maeGDP, maeGMP and finally the nucleoside VII.

N-2-(2′′-N′-methylanthraniloylaminoethyl)guanosine-5′-(β,γ-imido)triphosphate (X)

103 mg (0.22 mmol) N-2-(2′′-N′-methylanthraniloylaminoethyl)guanosine (VII) were phosphorylated to the non-hydrolysable β,γ-imidotriphosphate (maeGppNHp) in the same manner as described for maeGTP (IX) using the imidodiphosphate instead of the pyrophosphate. Since the imidodiphosphate is commercially available only as its sodium salt it must be converted into the DMF soluble tributylammonium salt by passing the sodiumimidodiphosphate (977 mg 2.24 mmol) through the strong acid cation-exchange resign Merck 1 (elution occured with methanol:H_2O 1:1 in a 1 l flask containing 4 ml tributylamine; the solvent was removed in vacuum and the residue was dried by coevaporation with dry DMF). The retention times of the maeGppNHp was 11.2 min. The yield of maeGppNHp (X) was 62 mg (40 %); ES-MS: m/z = 697 $(M-H)^-$.

maeGppNHp was dephosphorylated by phosphodiesterase to the maeGMP but appeared to be inert in the presence of alkaline phosphatase.

Proteins

H-Ras and Cdc42 were prepared as previously described[20, 21] and the nucleotide-free form of H-Ras was obtained as descibed by John et al.[2].

Fluorescence measurements

Static and slow time scale fluorescence measurements - excitation and emission fluorescence spectra and the dissociation of maeGppNHp from a stoichiometric complex with Ras in the presence of a 1000 fold excess of non-labelled GppNHp - were performed with a LS 50B Perkin-Elmer spectrofluorimeter as previously described[2, 18].

For higher time resolution fluorescence measurements - the association of mabGTP, maeGTP and maeGppNHp to Ras - a stopped-flow apparatus (Applied Photophysics SX16MV) was used (cut-off filter 408 nm)[2, 9]. Excitation and emission were measured at the respective maxima of the corresponding nucleotide; maeGTP (342 nm/433 nm), mabGTP (339 nm/428 nm) and maeGppNHp (333 nm/433 nm) respectively. The standard buffer was 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 2 mM DTE.

GTP-hydrolysis

The hydrolysis of mabGTP and maeGTP at the active site of Ras was assayed as a function of time by reversed-phase HPLC (Beckmann system Gold 166) under isocratic conditions in the presence of tetrabutylammonium bromide as described by Simon et al.[18]. 150 μM of a stoichiometric complex of Ras.mabGTP or Ras.maeGTP were incubated at 37 °C in standard buffer and aliquots were analyzed at appropriate time intervals. Data were fitted using the programm Grafit (Erithacus software).

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