Synthesis of GTP-Derived Ras Ligands

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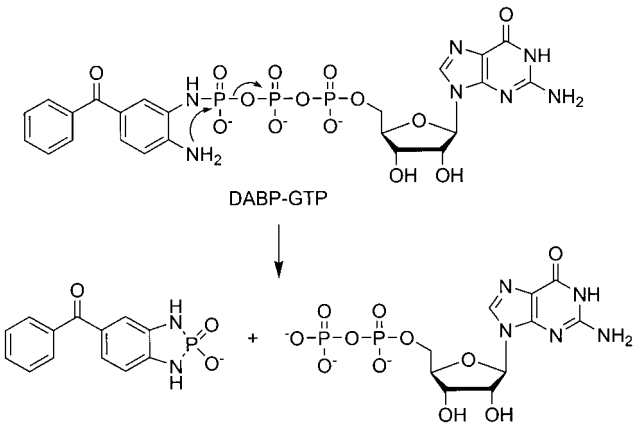
A practical and convenient method for the synthesis of acid- and base-sensitive GTP analogues carrying a further substituent at the terminal phosphate has been developed. Key to the successful synthesis of these potential ligands of the Ras protein is the use of Pd^0-sensitive allyl protecting groups in a one-pot synthesis that avoids evaporation steps. Initial biochemical analysis of a representative compound revealed that such GTP analogues can bind to Ras and might open up the possibility of developing small molecules that can act as deactivators of oncogenic Ras.

Introduction

GTP-binding proteins play major roles in signal transduction pathways that control cellular processes such as differentiation and proliferation.[1] In particular, proteins of the Ras superfamily act as molecular switches cycling between an inactive GDP-bound and an activated GTP-bound state. Ras proteins possess an intrinsic GTPase activity that is stimulated by GTPase-activating proteins (GAPs).[2] Impaired GTPase activity is typical for mutants of the Ras protein, which remain activated (that is, GTP-bound) and contribute to malignant transformation in approximately 30% of all human tumours.[3]

The development of small molecules that interfere with the transforming activity of Ras is of major interest to chemical biology and medicinal chemistry.[4] While approaches aiming at this goal have primarily focused on the inhibition of Ras farnesylation or inhibiting the interaction of Ras with its downstream effector Raf, very recently the development of molecules that bind to oncogenic Ras and induce hydrolysis of GTP to GDP was suggested as a new principle for Ras deactivation.[5–8] Specifically, diaminobenzophenone–GTP (DABP–GTP) was found to undergo aminolysis to give GDP when bound to oncogenic Ras (Scheme 1). This indicates that small-molecule deactivators of Ras might indeed be developed. However, DABP–GTP is not a suitable lead structure for the development of Ras-deactivating compounds, since its affinity for Ras is 400-fold lower than the affinity for the native ligand GTP and since it does not activate water to induce hydrolysis (as one would expect from an externally supplied Ras deactivator) but rather attacks the terminal phosphoric acid amide intramolecularly to form GDP (Scheme 1).



Scheme 1. *Cleavage of Ras-bound DABP–GTP through intramolecular aminolysis.*

Thus, in order to delineate structure parameters for the development of suitable candidate compounds, we embarked on the synthesis of DABP–GTP analogues that 1) bind to Ras, 2) do not undergo spontaneous or Ras-catalysed intramolecular aminolysis and 3) leave room for the subsequent introduction of functional groups that might induce GTP cleavage or binding to the surface of Ras close to the GTP-binding site.

Here we describe the development of a method for the flexible synthesis of such GTP derivatives and the preliminary investigation of three DABP analogues resulting in the identification of one compound that fulfils the criteria given above.

Results and Discussion

Synthesis

The synthesis of modified nucleotide triphosphates related to DABP–GTP is challenging, since these compounds are unstable under both acidic and basic conditions; this calls for the application of synthesis methods that proceed under very mild con-

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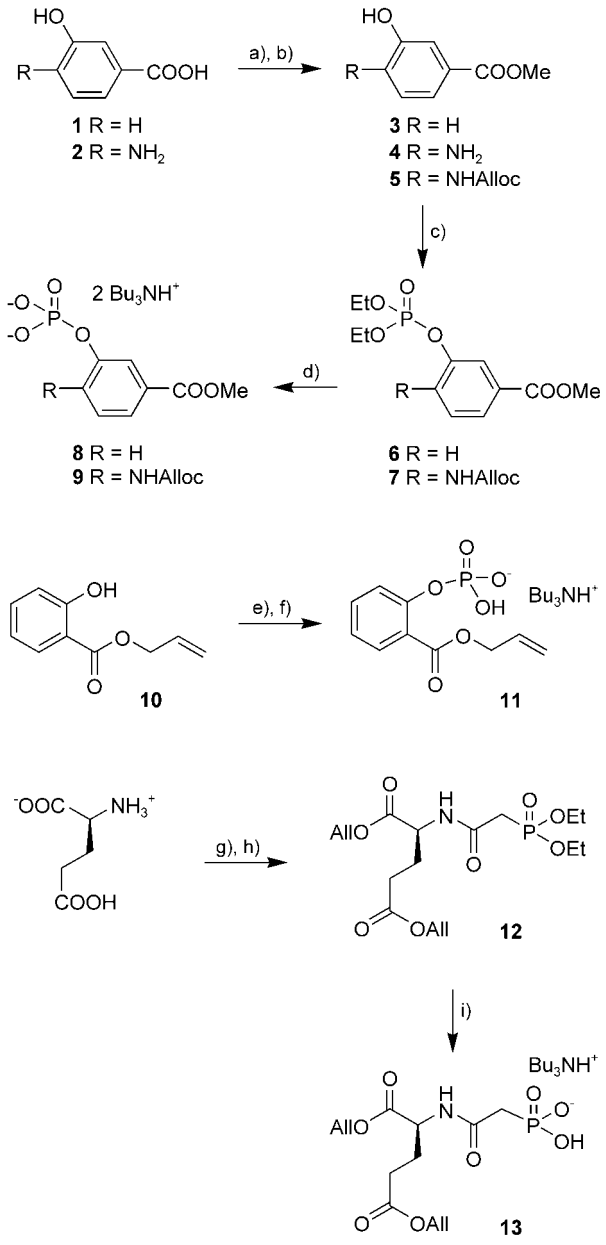
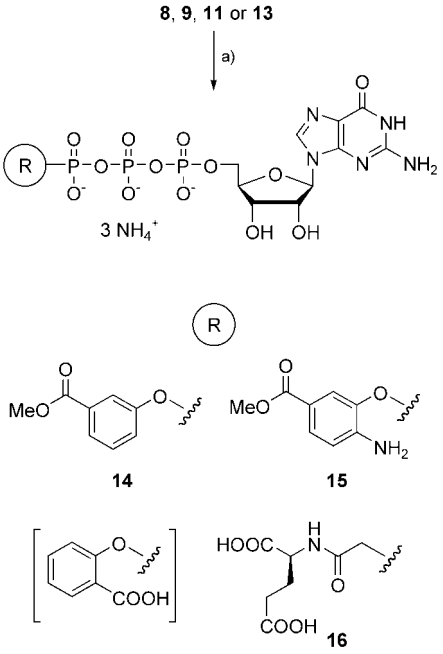
ditions.[9] We envisioned that the synthesis of target compounds **14**–**16** could be achieved by employing allylic protecting groups,[10] which can be cleaved selectively under weakly acidic or basic conditions for the construction of the functionalised triphosphate core. To this end, the phosphate building blocks **8** and **9** were synthesised from commercially available 3-hydroxybenzoic- (**1**) and 4-amino-3-hydroxybenzoic acid (**2**), respectively (Scheme 2). Both acids were protected in high yield as methyl esters by treatment with thionyl chloride in methanol. In the case of the 4-amino-3-hydroxybenzoic acid, the amino group was additionally protected with an allyloxycarbonyl (Alloc) protecting group under established conditions. The two masked phenol derivatives **3** and **5** were then phosphorylated with iodophosphate diethyl ester formed in situ by using iodine and triethylphosphite. In deviation from this method, initially described by Stowell et al.,[11] we employed *N*,*N*-dimethylaminopyridine (DMAP) instead of pyridine as base to prevent the formation of iodic acid and to activate the phenol groups. The reaction efficiently yielded the phosphorylated compounds **6** and **7** with 96% and 88% yield, respectively. Both were deprotected by treatment with trimethylsilyl bromide and subsequent hydrolysis. The resulting phosphates were converted to the tributylammonium salts **8** and **9** to increase their solubility in organic solvents. The salicylic acid-derived phosphate building block **11** was synthesised by employing a similar sequence (Scheme 2). Allyl ester **10** was obtained from the acid by treatment with thionyl chloride in allyl alcohol. The L-glutamic acid derived phosphonate building block **13** was prepared starting from the corresponding amino acid by protection as a bisallyl ester and subsequent coupling to diethylphosphonoacetic acid (Scheme 2). Deprotection of the phosphonate was achieved as described above, and the resulting phosphonate was converted into the monobutylammonium salt **13**.



Scheme 2. *Synthesis of phosphate and phosphonate building blocks* ***8***, ***9***, ***11*** *and* ***13****. a) 3-hydroxybenzoic acid (****1****) or 4-amino-3-hydroxybenzoic acid (****2****), $SOCl_2$, MeOH, RT, 3 h, 97% (****3****), 93% (****4****); b)* ***4****, allyl chloroformate, pyridine, THF, RT, 1 h 30, 45% (****5****); c)* ***3*** *or* ***5****, I_2, $P(OEt)_3$, DMAP, CH_2Cl_2, 0°C→RT, 1 h, 96% (****6****) or 88% (****7****); d)* ***6*** *or* ***7****, $(CH_3)_3SiBr$, RT, 18 h, 79% (****8****) or 72 h, 90% (****9****); e) I_2, $P(OEt)_3$, DMAP, CH_2Cl_2, 0°C→RT, 1 h, 82%; f) $(CH_3)_3SiBr$, RT, 18 h, 70%; g) L-glutamic acid, $SOCl_2$, AllOH, Δ, 3 h, 57%; h) diethylphosphonoacetic acid, benzotriazol-1-yl-N-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIEA), DMF, RT, 6 h, 41%; i) $(CH_3)_3SiBr$, RT, 18 h, 98%.*

Building blocks **8**, **9**, **11** and **13** were subjected to coupling with GDP by employing imidazolyl-GDP as activated reagent in *N*,*N*-dimethylformamide as solvent. To this end, GDP was converted to the tributylammonium salt by using a DOWEX 50WX2-100 ion-exchange resin and tributylamine and then activated with 1,1′-carbonyldiimidazole.[12] The initial coupling products were not isolated but rather the allylic protecting groups were removed directly in a convenient one-pot Pd^0-mediated deallylation procedure in the presence of a dimethylamine–borane complex (Scheme 3).[13] The efficiency of this deprotection and thereby the success of the subsequent product isolation critically depends on the choice of the allyl-cation trapping nucleophile. As a preliminary experiment to evaluate the relative efficiency of several scavengers, we monitored the Alloc removal using RP-C18 HPLC analysis for the phosphate building block **7**, which was taken as reference compound (see the Experimental Section). This study revealed that the deprotection is quantitative and complete within 10 minutes when using the dimethylamine–borane complex, whereas 80% yield was obtained within the same reaction time if *N*-methyl aniline or morpholine were employed. No improvement was observed after an hour. *N*,*N*′-dimethyl barbituric acid was found to be less potent than the amine–borane complex but more efficient than *N*-methyl aniline and morphline, with 75% conversion after 10 minutes and up to 90% after an hour. Due to the efficiency of the deprotection reaction with the amine–borane complex, a one-pot procedure could be developed that gave access to compounds **14**–**16** in pure form after one simple ion-exchange chromatography step. Attempts to isolate the products after evaporation of the solvent led to extensive decomposition of the triphosphates. This problem could be over-



Scheme 3. *Synthesis of GTP derivatives **14**–**16**. a) imidazolyl-GDP, DMF, 50 °C, 24 h; Pd(PPh$_3$)$_4$, Me$_2$N·BH$_3$, DMF, RT, 1 h, 39% (**14**); 47% (**15**); 26% (**16**). Note: compound **11** did not provide the expected phosphoester derivative.*

come by means of a precipitation/centrifugation step. Finally, compounds **14**, **15** and **16** were isolated with acceptable yields ranging from 26% to 47%. Unexpectedly, the phosphoester derivative derived from 2-hydroxybenzoic acid turned out to be very unstable and could not be isolated (compound shown in brackets in Scheme 3). Rather, the corresponding phosphate building block **11** and GDP were recovered by ion-exchange chromatography and characterised by using ^{31}P NMR. In order to ensure that the initial coupling was successful and to confirm the instability of this compound, we prepared and purified the corresponding protected allyl ester triphosphate nucleotide in 51% yield (not shown). Deallylation attempts led to the same result, that is, decomposition presumably due to nucleophilic attack of the *ortho* carboxylate group on the γ-phosphate to give a mixed anhydride that is then hydrolysed to the deprotected phosphate building block and GDP. This assumption is in accordance with the results of previous studies on the hydrolysis mechanism of *bis*(2-carboxyphenyl) phosphate.[14]

Binding of the GTP analogues to the Y32W Ras mutant

Compound **15** embodies a potentially nucleophilic amino group *ortho* to the site of attachment to the GTP core and an ester *para* to the amino function. Thus, it is a relatively close analogue of DABP–GTP although it is not a phosphoramidate. In order to determine whether this compound is cleaved like DABP–GTP via intramolecular aminolysis when bound to oncogenic Ras, its stability in the presence of the Y32W Ras mutant either in the nucleotide-bound or the nucleotide-free form was investigated by using fluorescence spectroscopy[15,16] and RP-HPLC.[17] Cleavage of GTP analogues in the presence of this Ras mutant, which has been extensively used as a Ras wild-type reference protein,[18] can be monitored by the increase of the fluorescence intensity of the Y32W Ras mutant, which occurs as result of the conformational switch from the GTP-bound state to the GDP-bound state. In the RP-HPLC experiments, aliquots taken from an incubation solution of Y32W Ras and GTP analogues at different time intervals were analysed. Both analytical methods revealed that compound **15** was stable in the presence of Y32W Ras. Not only is the compound significantly more stable than DABP–GTP in the presence of the protein, it is also stable in the presence of magnesium ions for at least one day whereas DABP–GTP is fully hydrolysed within 5 hours under the same conditions.[7] This stability in the presence of the protein might be explained by the inability of compound **15** to bind to the Ras mutant employed. However we characterised the complex between Y32W Ras and compound **15** by MALDI mass spectrometry (Figure 1). The MALDI-MS spectrum

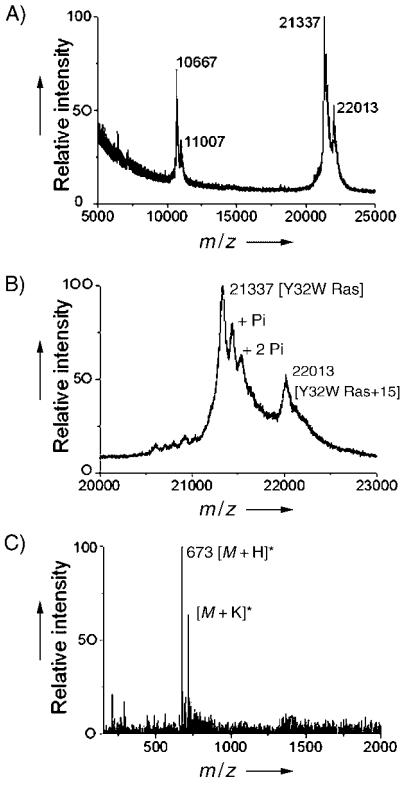


Figure 1. *Mass spectrometric analysis of complex formation between Y32W Ras and compound **15**. A) MALDI-MS spectrum of the complex between Ras and the compound **15**. B) Magnification of the region of singly charged molecular ions. C) ESI mass spectrum of compound **15**.*

of the enzyme alone (not shown) shows the singly charged molecular ion (21337 Da) and other signals corresponding to singly charged molecular ions of enzyme adducts with inorganic phosphate. Doubly charged molecular ions (10667 Da) were also observed. MALDI-MS analysis of the enzyme incubated with compound **15** shows the same ions and, in addition, doubly or singly charged molecular ions with masses of 11007 and 22013 Da, respectively, corresponding to the complex

(Figure 1A and B). The difference between the molecular masses of the complex and the enzyme is 676 Da, which corresponds within the error of the instrument to the molecular mass of compound **15** as determined by ESI-MS (Figure 1C). Considering the accuracy of the MALDI-MS technique for proteins, this result clearly indicates that the complex was formed and that compound **15** binds to the Ras mutant employed. In addition, even if the affinity of GTP analogues for Ras is not as high as the value recorded for the natural ligand itself when exposed to nucleotide-free Ras, the basic affinity mediated predominantly by the GTP structure ensures that such compounds still bind with significant affinity.[6,19] Finally, the binding affinities of analogues **14** and **15** to Ras were determined by displacing the fluorescent 2′(3′)-*O*-(*N*-methylanthraniloyl)-GDP (mant-GDP) bound to Ras with increasing concentrations and fitting the data with a three-component binding model (Figure 2). The fluorescence decrease is saturable where the final fluorescence reaches the value of free mant-GDP. Comparison with mant-GDP[20] gives equilibrium dissociation constants of 40 pM for **14** and 33 pM for **15**; this indicates that the affinity is similar than that of GDP, which was used as a control (K_d = 16 pM). DABP–GTP spontaneously decomposes in the Ras standard buffer through an aminolysis process, that is, by nucleophilic attack of the *ortho* amino group on the electrophilic γ-phosphate.[7] The different reactivity of compound **15** might be due to a lower electrophilic character of the γ-phosphate in solution and in complex with Ras, since DABP–GTP incorporates a phosphoric acid amide whereas, in **15**, the substituent is linked to GTP via a more stable phosphoric acid ester bond. In addition, in the case of DABP–GTP, a very stable cyclic phosphodiamidate is formed (Scheme 1) that also drives the overall reaction.

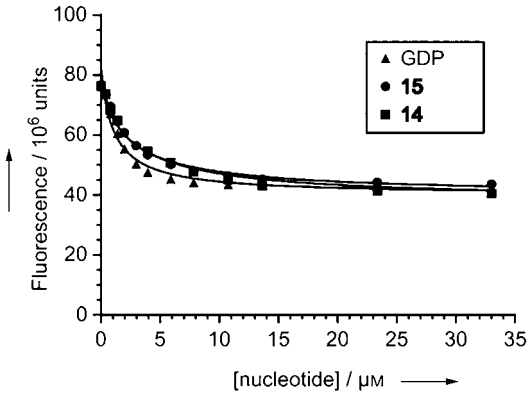


Figure 2. *Ras-bound mant-nucleotide was displaced by addition of the indicated amounts of either GDP or nucleotide analogues. The observed fluorescence upon equilibration was fitted as described in the Experimental Section. The following K_d values for the Ras interaction were obtained: GDP: 16 pM, **15**: 33 pM, **14**: 40 pM.*

Conclusion

In conclusion we have developed a convenient method for the synthesis of GTP analogues that makes use of allylic protecting groups as the key methodology. While the purpose of our investigation was focused primarily on the establishment of such a synthetic method, preliminary biochemical investigation of a close analogue of the guiding DABP–GTP ligand indicates that, indeed, compounds may be identified that meet the criteria raised above. Thus DABP analogue **15** binds to Ras, but does not undergo undesired cleavage through intramolecular aminolysis in the presence of a Ras mutant; this opens up the possibility of introducing further substituents either in the aromatic ring or through a carboxylic acid derivative. Such functional groups or structural units might mediate binding of possible Ras deactivators derived from DABP–GTP analogues on the surface of the protein close to the GTP binding site, and they might serve to induce GTP hydrolysis on oncogenic Ras. The pronounced sensitivity of the GTP conjugate expected from **11** suggests that, for instance, a carboxylic acid that can act as a nucleophile may serve this purpose.

Experimental Section

General: Optical rotations were measured with a Perkin–Elmer 241 or 341 polarimeter. TLC was performed on Kieselgel 60F_{254} (Merck). Flash chromatography was performed on silica gel (230–400 mesh). NMR spectra were recorded with a Varian Mercury 400 machine. The signal of the residual protonated solvent ($CDCl_3$ or D_2O) was taken as reference [^{1}H: δ = 7.26 ($CHCl_3$) or 4.79 (D_2O), ^{13}C: δ = 77.0 ($CHCl_3$)]. Mass spectra were recorded on the following spectrometers: FAB = Finnigan MAT MS 70 (3-nitrobenzylalcohol (NBA) as matrix), MALDI-TOF = Perseptive Biosystems Voyager BioSpectrometry Workstation or Voyager-DE Pro BioSpectrometry™ Workstation (2,5-dihydroxy-benzoic acid (DHB) or sinapinic acid as matrix), ESI = Finnigan Thermoquest LCQ. Recombinant H-Ras mutants were expressed from *Escherichia coli* as described by Tucker et al.[21] Nucleotide-free protein was prepared as previously described.[15]

General procedure for the synthesis of methyl or allyl ester derivatives: Thionyl chloride (15 mmol) was added dropwise at 0 °C to a solution of acid derivatives (10 mmol) in methanol or allyl alcohol (15 mL). The solution was then stirred at room temperature (MeOH) or refluxed (AllOH) for 5 h. The solvent was evaporated, and the residue was dissolved in EtOAc and washed with a saturated aqueous solution of $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure to give methyl or allyl ester derivatives.

General procedure for phosphorylation: Iodine (2.5 equiv) was added at 0 °C to a solution of triethylphosphite (2.7 equiv) in dry CH_2Cl_2 (70 mL). After 10 min at 0 °C, the solution was allowed to warm up to room temperature and added dropwise to a solution of the corresponding benzoic acid ester derivative (1 equiv) and DMAP (2.5 equiv) in dry CH_2Cl_2 (60 mL) at 0 °C. This solution was stirred at 0 °C for 30 min and warmed to room temperature. After 30 min, the solution was washed with water and brine. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The phosphorylated compounds were purified by flash chromatography.

4-Allyloxycarbonylamino-3-(diethoxyphosphoryloxy)benzoic acid methyl ester (**7**) (selected data): orange solid (88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.27 (td, J = 6.8 Hz, $J_{H\text{-}P}$ = 1.2 Hz, 6H; CH_2CH_3), 3.79 (s, 3H; OCH_3), 4.11–4.21 (m, 4H; CH_2CH_3), 4.59 (dt, J = 5.6, 1.6 Hz, 2H; OCH_2CHCH_2), 5.17 (dq, J = 10.4, 1.6 Hz, 1H;

OCH_2CHCH_2), 5.28 (dq, $J=17.2$, 1.6 Hz, 1H; OCH_2CHCH_2), 5.87 (tdd, $J=5.6$ Hz, $J=17.2$ and $J=10.4$ Hz, 1H; OCH_2CHCH_2), 7.70 (s, 1H; NH), 7.76 (dd, $J=8.4$, 1.2 Hz, 1H; H6-Ar), 7.81 (dd, $J=J_{H\text{-}P}=1.2$ Hz, 1H; H2-Ar), 8.13 (d, $J=8.4$ Hz, 1H; H5-Ar); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=15.9$ (d, $J_{C\text{-}P}=6.9$ Hz, $-CH_2CH_3$), 51.9 ($-OCH_3$), 65.1 (d, $J_{C\text{-}P}=5.8$ Hz, $-CH_2CH_3$), 66.0 (CH_2, allyl), 118.1 ($=CH_2$), 119.1 (C2-Ar), 121.2 (C5-Ar), 124.5 (C1-Ar), 127.0 (C6-Ar), 131.7 ($CH=CH_2$), 133.9 (d, $J_{C\text{-}P}=5.6$ Hz, C4-Ar), 138.4 (d, $J_{C\text{-}P}=6.6$ Hz, C3-Ar), 152.3 (C=O), 165.3 (C=O, ester); ^{31}P NMR (160 MHz, $CDCl_3$): $\delta=-4.3$; HR-MS (FAB-MS): *m/z*: calcd for $C_{16}H_{22}NO_8P$: 388.1161 $[M+H]^+$; found: 388.1150.

General procedure for phosphate deprotection: A solution of the protected phosphate in trimethylsilyl bromide (8 equiv) was stirred for 72 h. The trimethylsilyl bromide was evaporated under reduced pressure. The residue was dissolved in Et_2O and extracted with water. Tributylamine (10 equiv) was then added, and the solution was stirred for 5 min and decanted. The aqueous layer was lyophilised to give the phosphate building blocks.

4-Allyloxycarbonylamino-3-phosphonooxy-benzoic acid methyl ester, bis(tributylammonium) salt (**9**) (selected data): yellow oil (90%). ^1H NMR (400 MHz, $CDCl_3$): $\delta=0.87$ (t, $J=7.2$ Hz, 18H; $nBuCH_3$), 1.21–1.30 (m, 12H; $-CH_2CH_2CH_2CH_3$); 1.48–1.56 (m, 12H; $-CH_2CH_2CH_2CH_3$), 2.67–2.71 (m, 12H; $-CH_2CH_2CH_2CH_3$), 3.80 (s, 3H; OCH_3), 4.59 (dt, $J=5.6$, 1.6 Hz, 2H; OCH_2CHCH_2), 5.18 (dq, $J=10.4$, 1.6 Hz, 1H; OCH_2CHCH_2), 5.32 (dq, $J=17.2$, 1.6 Hz, 1H; OCH_2CHCH_2), 5.87 (tdd, $J=5.6$, 17.2, 10.4 Hz, 1H; OCH_2CHCH_2), 6.10 (br, 2H; Bu_3NH^+), 7.67 (dd, $J=8.4$, 1.2 Hz, 1H; H6-Ar), 7.89 (dd, $J=J_{H\text{-}P}=1.2$ Hz, 1H; H2-Ar), 8.07 (d, $J=8.4$ Hz, 1H; H5-Ar), 9.63 (s, 1H; NH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=13.8$ ($nBuCH_3$), 20.3 ($-CH_2CH_2CH_2CH_3$), 25.6 ($-CH_2CH_2CH_2CH_3$), 51.6 (OCH_3), 51.7 ($-CH_2CH_2CH_2CH_3$), 65.6 (CH_2, allyl), 117.8 ($=CH_2$), 118.7 (C2-Ar), 122.9 (C5-Ar), 124.0 (C6-Ar), 124.7 (C1-Ar), 132.4 ($CH=CH_2$), 135.8 (d, $J_{C\text{-}P}=3$ Hz, C4-Ar), 142.8 (d, $J_{C\text{-}P}=6.7$ Hz, C3-Ar), 153.3 (C=O), 166.5 (C=O, ester); ^{31}P NMR (160 MHz, $CDCl_3$): $\delta=-0.5$; MALDI-MS (DHB): *m/z*: 354 $[M+Na]^+$.

General procedure for the synthesis of substituted GTP analogues: GDP disodium salt (0.5 g, 1.026 mmol) was converted to GDP tri-*n*-butylammonium salt by using DOWEX 50WX2-100 ion-exchange resin (15 g). *N*,*N*-Carbonyl diimidazole (CDI; 0.04 g, 0.25 mmol, 5 equiv) was added to a solution of GDP tri-*n*-butylammonium salt (0.05 g, 0.05 mmol, 1 equiv) in DMF (2 mL). The solution was stirred at room temperature for 6 h, and methanol (0.0064 g, 0.2 mmol, 4 equiv) was added to quench the excess of CDI. The solution was then stirred for 30 min at room temperature and an additional 30 min under vacuum. The resulting solution was added to a solution of the phosphate building block (**9**; 0.07 g, 0.1 mmol, 2 equiv) in DMF (1 mL). After 24 h at 50°C, the solution was cooled to room temperature and purged with argon. Tetrakis(triphenyl phosphine)palladium (0.011 g, 9.5 μmol, 0.2 equiv) and the dimethylamino–borane complex (0.147 g, 2.5 mmol, 50 equiv) were added consecutively. After 1 h, cold diethyl ether (50 mL) was added, and the precipitated residue was collected by centrifugation. The white solid was dissolved in water. The solution was centrifuged to remove insoluble material and was then loaded on a Q sepharose column. The desired compound was eluted with a stepping gradient of an ammonium carbonate buffer from 0.025 M to 0.6 M in 0.025 increments (pH 8, fraction volume of 10 mL). Appropriate fractions (UV analysis) were pooled and lyophilised to give the substituted GTP derivate.

(3-Hydroxybenzoic acid methyl ester) guanosine 5′-triphosphate, triammonium salt (**14**): white solid (39%).$[\alpha]_D^{20}=-13°$ ($c=0.1$ in H_2O); ^1H NMR (400 MHz, D_2O): $\delta=3.85$ (s, 3H; $-OCH_3$), 4.19–4.30 (m, 3H; $H_{5'}$, $H_{4'}$), 4.44 (t, $J=4.5$ Hz, 1H; $H_{3'}$), 4.55 (t, $J=4$ Hz, 1H; $H_{2'}$), 5.78 (d, $J=4$ Hz, 1H; $H_{1'}$), 7.30–7.34 (m, 1H; H-Ar), 7.41–7.43 (m, 1H; H-Ar), 7.53–7.59 (m, 2H; H-Ar), 8.07 (s, 1H; H8); ^{13}C NMR (100 MHz, D_2O): $\delta=53.9$ ($-OCH_3$), 66.1 (d, $J_{C\text{-}P}=5.3$ Hz, $C_{5'}$), 71.0 ($C_{3'}$), 75.2 ($C_{2'}$), 84.4 (d, $J_{C\text{-}P}=6.1$ Hz, $C_{4'}$), 88.8 ($C_{1'}$), 115.8 (C5), 122.1 (d, $J_{C\text{-}P}=5.2$ Hz, C2-Ar), 125.8 (C6-Ar), 126.5 (d, $J_{C\text{-}P}=3.1$ Hz, C4-Ar), 130.8 (C5-Ar), 131.4 (C1-Ar), 137.9 (C8), 152.5 (C4), 154.7 (C2), 158.7 (C6), 158.9 (d, $J_{C\text{-}P}=4.4$ Hz, C3-Ar), 169.0 (C=O, ester); ^{31}P NMR (160 MHz, D_2O): $\delta=-9.1$ (d, $J=18.5$ Hz, 1P; γP), −14 (d, $J=19.5$ Hz, 1P; αP), −21.0 (t, $J=19$ Hz, 1P; βP); ESI-MS: *m/z*: 658 $[M+H]^+$; RP-C18 HPLC (100 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 10 mM tetrabutylammonium bromide, 16.5% acetonitrile): $t_R=4.17$ min.

(4-Amino-3-hydroxybenzoic acid methyl ester) guanosine 5′-triphosphate, triammonium salt (**15**): White solid (0.017 g, 47%). $[\alpha]_D^{20}=-27°$ ($c=0.072$ in H_2O); ^1H NMR (400 MHz, D_2O): $\delta=3.79$ (s, 3H; $-OCH_3$), 4.19–4.31 (m, 3H; $H_{5'}$, $H_{4'}$), 4.44 (t, $J=4$ Hz, 1H; $H_{3'}$), 4.52 (t, $J=4$ Hz, 1H; $H_{2'}$), 5.77 (d, $J=4$ Hz, 1H; $H_{1'}$), 6.73 (dd, $J=8.4$, 2.8 Hz, 1H; H-Ar), 7.37 (m, 1H; H-Ar), 7.57 (m, 1H; H-Ar), 8.18 (s, 1H; H8); ^{13}C NMR (100 MHz, D_2O): $\delta=52.6$ ($-OCH_3$), 64.9 (d, $J_{C\text{-}P}=5.5$ Hz, $C_{5'}$), 69.8 ($C_{3'}$), 74.4 ($C_{2'}$), 83.3 (d, $J_{C\text{-}P}=4.8$ Hz, $C_{4'}$), 88.1 ($C_{1'}$), 113.9 (C5), 116.1 (C2-Ar), 118.8 (C5-Ar), 121.4 (C6-Ar), 126.9 (C1-Ar), 136.6 (C8), 138.5 (d, $J_{C\text{-}P}=6.9$ Hz, C3-Ar), 142.9 (C4-Ar), 150.3 (C4), 153.9 (C2), 157.3(C8), 168,4 (C=O, ester); ^{31}P NMR (160 MHz, D_2O): $\delta=-10.0$ (d, $J=17.4$ Hz, 1P; γP), −13.9 (d, $J=17.6$ Hz, 1P; αP), −21.5 (br, 1P; βP); ESI-MS: *m/z*: 673 $[M+H]^+$; RP-C18 HPLC (100 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 10 mM tetrabutylammonium bromide, 16.5% acetonitrile): $t_R=4.39$ min.

(L-glutamic acid) triphosphate nucleotide derivative, triammonium salt (**16**): white solid (26%). $[\alpha]_D^{20}=-14°$ ($c=0.1$ in H_2O); ^1H NMR (400 MHz, D_2O): $\delta=1.96$–2.05 (m, 1H; Hβ), 2.15–2.24 (m, 1H; Hβ), 2.54 (t, $J=7.4$ Hz, 2H; Hγ), 2.98 (d, $J=21$ Hz, 2H; CH_2P), 4.15–4.34 (m, 3H; $H_{5'}$, $H_{4'}$), 4.41–4–46 (m, 1H; $H_{3'}$), 4.54 (t, $J=4.4$ Hz, 1H; $H_{2'}$), 4.74 (t, $J=4.8$ Hz, 1H; Hα), 5.98 (d, $J=4.5$ Hz, 1H; $H_{1'}$), 8.23 (s, 1H; H8); ^{13}C NMR (100 MHz, D_2O): $\delta=26.7$ and 30.9 (Cβ and Cγ), 38.0 (d, $J_{C\text{-}P}=125$ Hz, CH_2P), 53.3 (Cα), 64.5 (d, $J_{C\text{-}P}=5.5$ Hz, $C_{5'}$), 70.4 ($C_{3'}$), 74.2 ($C_{2'}$), 84.0 (d, $J_{C\text{-}P}=4.8$ Hz, $C_{4'}$), 87.7 ($C_{1'}$), 114.7 (C5), 137.3 (C8), 151.3 (C4), 154.2 (C2), 158.0 (C6), 171.1 (C=O, amide), 176.1 and 177.6 (C=O, acid); ^{31}P NMR (160 MHz, D_2O): $\delta=7.4$ (d, $J=24$ Hz, 1P; γP), −9.9 (d, $J=18$ Hz, 1P; αP), −21.5 (t, $J=20$ Hz, 1P; βP); ESI-MS: *m/z*: 695 $[M+H]^+$; RP-C18 HPLC (100 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 10 mM tetrabutylammonium bromide, 16.5% acetonitrile): $t_R=2.77$ min.

Monitoring of the Alloc removal by RP-C18 HPLC: The scavenger (*N*-methyl aniline, morpholine, barbituric acid or $(CH_3)_2NH{\cdot}BH_3$, 20 equiv) was added to a solution of **7** (5 mg, 12.9 μmol) and $Pd(PPh_3)_4$ (1.49 mg, 1.29 μmol) in THF (250 μL). At different time points, aliquots (25 μL) were taken and evaporated under high vacuum. The mixture was dissolved in acetonitrile (1 mL) and analysed by RP-C18 HPLC (injection volume = 5 μL, column: Macherey Nagel Nucleodur® C18 Garavity 3 μm CC 125/4, flow rate = 1 mL min^{-1}, Solvent A: 0.1% TFA in water, B: acetonitrile, linear gradient: 20% to 100% B in 10 min). Compound **7**: $t_R=7.44$ min; deprotected: $t_R=5.81$ min.

Ras-GTPase assays: RP-C18 HPLC experiments: GTP analogues (50 μM) were incubated at 30°C with or without nucleotide-free Y32W Ras (67.7 μM) in the Ras standard buffer (30 mM Tris/HCl (pH 7.5), 3 mM dithioerythritol, 5 mM $MgCl_2$). Sodium orthovanadate (1 mM) was added as inhibitor of possible phosphatase contaminations of the Ras proteins.[15] Aliquots taken at different time points were quickly analysed by HPLC on an ODS-Hypersil C18 column (5 μm bead size, 0.46×25 cm, Beckman/USA) under isocrat-

ic conditions (100 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 10 mM tetrabutylammonium bromide, 16.5% acetonitrile, flow rate 1.8 mL min^{-1}).[22] From the peak areas, the stability of GTP analogues was determined in the absence or the presence of the enzyme.

Fluorescence spectroscopy: hydrolysis of GTP analogues (1 μM) was monitored on a LS50B Perkin–Elmer spectrofluorometer with Y32W Ras (1 μM) in the standard buffer (excitation: 295 nm, emission: 350 nm).

Mass spectrometry: MALDI-MS analyses were performed on a solution of Y32W Ras (1 μM) and compound **15** (1 μM) in the standard buffer (1 μL) diluted with the matrix (sinapinic acid solution, 1 μL).

K_d determination: The standard buffer for all reactions was Tris (pH 7.5, 30 mM), $MgCl_2$ (5 mM) and dithioerythritol (3 mM). Ras(Y32W) was loaded with mant-GDP by incubating the nucleotide-free protein[15] with an excess of the nucleotide. Unbound nucleotide was removed by size-exclusion chromatography on a NAP5 column (Amersham-Pharmacia, Freiburg). A 500 μL reaction containing Ras(Y32W)·mant-GDP (1 μM) and His-tagged exchange factor SOS (amino acids 549–1049; 2 μM) was analysed at 20 °C in a FluoroMax II fluorescence spectrometer (excitation wavelength 366 nm, measured emission at 450 nm). Ras-bound mant nucleotide was displaced by increasing concentrations of nucleotide (GDP, **14**, **15**), and the reaction was followed by the decrease in fluorescence. K_d values for the nucleotide were determined with the program Scientist (MicroMath, Salt Lake City, USA) by fitting the obtained fluorescence transients to a competitive three-component binding model. (Independent variable: $[compound]_{total}$; dependent variables: $[Ras]_{free}$, $[mant\text{-}GDP]_{free}$, $[compound]_{free}$, [Ras*-mant-GDP], [Ras*compound], fluorescence; free parameters: K_d(Ras*compound), fixed parameters: K_d(Ras*mant-GDP), $[Ras]_{total}$, $[mant\text{-}GDP]_{total}$, fluorescence ($mant\text{-}GDP_{free}$), fluorescence (Ras*-mant-GDP); square brackets denote concentrations. Ras binds either mant-GDP or the compound according to the law of mass action, but not both molecules simultaneously. The K_d(Ras*mant-GDP) value used was 13 pM.[20]

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