Interaction of GTP Derivatives with Cellular and Oncogenic *ras*-p21 Proteins

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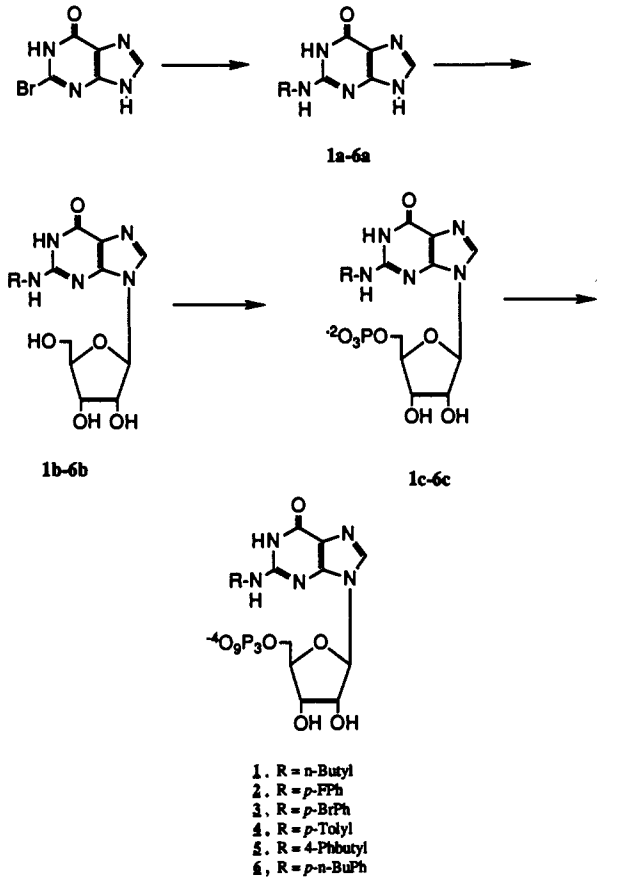
A series of N^2-substituted guanosine 5'-triphosphates was synthesized from the corresponding nucleosides. The nucleosides were prepared by treatment of N^2-substituted guanines with tetra-*O*-acetylribose under conditions which maximized the yield of the 9-β-guanosine isomers. These nucleotides and several sugar- and base-modified analogues of GTP were tested for their ability to bind to cellular and oncogenic forms of the GTP/GDP binding proteins, Ha-*ras*-p21. Several N^2-substituted GTPs showed affinities higher than that of GDP itself, and the N^2-[*p*-(*n*-butyl)phenyl] derivative bound to the oncogenic mutant, Leu-61 p21, twice as strongly as to the cellular protein. Changes in relative affinities of the nucleotides are discussed with reference to reported crystallographic structures of p21.

ras refers to a family of structurally related genes that have been highly conserved in a wide spectrum of eukaryotic cells.[1-3] Mammalian *ras* genes code for 21 000-Da membrane-associated proteins, termed p21, which are typically 188 or 189 amino acids in length. The *ras* proteins, in common with members of a large family of "G" proteins, recognize and bind guanine ribonucleotides (5'-di- and triphosphates) and possess intrinsic, but low, GTPase activity.[4-6] The cytosolic factor GAP (GTPase-activating protein) stimulates the GTPase of normal *ras* p21 by 100-fold in *Xenopus* oocytes in culture.[7] *ras* genes appear to be targets of genetic lesions resulting in single amino acid substitutions in p21, particularly in positions 12 and 61, and these mutations are often accompanied by a reduction of intrinsic GTPase activity and loss of sensitivity to GAP.[8] In addition, certain retroviruses carry mutated *ras* genes which are able to transform mammalian cells in culture and in vivo. These subtle alterations are sufficient to confer oncogenic properties to the *ras* proteins, and corresponding mutated *ras* genes are highly transforming to cells in culture.[9,10] Mutations in *ras* genes have been identified in a significant percentage of human cancers, and these findings have prompted suggestions that these genes or their protein products may be targets for anti-*ras* antitumor drugs.[11]

By analogy with the transducing roles of other G proteins, oncogenic p21 may be part of a signal-transducing complex that has lost its growth regulatory capacities. A strong homology exists among G proteins, especially in sequences that form the nucleotide binding domain. The detailed picture of the GTP/GDP site recently afforded by X-ray crystallography allows interpretation of affinities of nucleotide analogues to p21 at the functional group level.[12,13] Indeed, effects of modification of the ligand structure as well as that of the proteins (by site-directed mutagenesis) have substantially confirmed models of the GTP/GDP site at the level of specific atomic interactions.[14]

We have begun a search for non-nucleotide compounds that may bind and prevent activation of oncogenic forms of p21. Such compounds should be able to enter cells, be nonintrusive in critical biochemical pathways of nucleotide metabolism, and seek out the specific mutated p21 and bind it selectively. As a first approach to this problem we have tested several GTP derivatives for their ability to compete with [^{3}H]GDP for binding to the normal cellular and an oncogenic mutant form of p21. We first want to determine essential requirements for ligand binding, and equally importantly, structural variations that may enhance affinity of analogues to the oncogenic form of p21. The results of an earlier study with the retinal G protein transducin indicated that the modified GTP derivative N^2-(*p*-*n*-butylphenyl)guanosine 5'-triphosphate (BuPGTP, 6) could bind to transducin with an affinity nearly equal to that of GTP, serve as a substrate for the GTPase ac-

Scheme I



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tivity of the protein, and activate, in rod outer segments, the cyclicGMP phosphodiesterase that is the intracellular "target" of the transducin·GTP complex.[15] Therefore, we synthesized a series of N^2-substituted GTPs, and, in this paper, describe the ability of these and other GTP derivatives to bind to *ras*-p21 proteins.

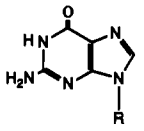
Chemistry

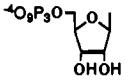
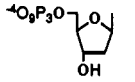
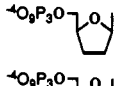
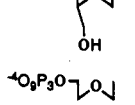
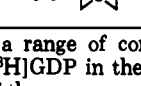
The N^2-substituted guanosine 5′-triphosphates **1–6** were prepared as outlined in Scheme I. The N^2-substituted guanines **1a–6a** were obtained by reacting 2-bromohypoxanthine with the appropriate amine in refluxing 2-methoxyethanol, as reported previously for the synthesis of analogous compounds.[16] The 9-β-D-ribofuranosyl derivatives of **1a–6a**, compounds **1b–6b**, were synthesized by the treatment of the trimethylsilylated bases with tetra-*O*-acetylribose in dry acetonitrile in the presence of trimethylsilyl trifluoromethanesulfonate as catalyst, as previously reported for the synthesis of **4b**[16] and **6b**.[17] The reaction conditions were selected to maximize conversion to the more stable 9-β-isomers,[18] which were purified in the protected (acetylated) form from the minor reaction components, most likely the 7-β-ribofuranosyl derivatives, by fractional crystallization.[17] Deblocking of the products with methanol saturated with ammonia gave the desired nucleosides **1b–6b** in ca. 60% yields.

In addition to the formation of only β-anomeric products, we[16–18] and others[19] have shown that this glycosylation method promotes conversion of kinetically favored N-7 ribonucleosides into the thermodynamically favored N-9 ribonucleosides of guanine derivatives. The identity of new compounds **1b–3b** and **5b** as 9-β-ribonucleosides was confirmed by their characteristic ^{1}H NMR spectra (see the Experimental Section). As reported for **6b** and its 7-β-isomer,[20] the chemical shift of H-1′ is about 0.25 ppm downfield and that of H-2′ is about 0.1 ppm upfield in the 7-β compared to the 9-β-isomers, and all other ribose ring ^{1}H chemical shifts and coupling constants are essentially identical between the isomers. In addition, N-7 substitution causes the purine ring 8-H resonance to shift downfield about 0.35 ppm relative to N-9 substitution.[20] The near identity of characteristic ^{1}H NMR chemical shifts of the products with those of **4b**[16] and **6b**[20] confirm that they are 9-β-ribofuranosyl isomers.

Nucleosides **1b–6b** were converted to the 5′-phosphates **1c–6c** by treatment with phosphoryl chloride in trimethyl phosphate as described.[21] The desired 5′-triphosphates, compounds **1–6**, were obtained by condensation of **1c–6c**, previously activated with 1,1′-carbonyldiimidazole, with tributylammonium pyrophosphate in hexamethylphosphoramide.[22] All nucleotides were purified by anion exchange HPLC using an AX-100 column, and elution with ammonium bicarbonate/acetonitrile gave the triphosphates as ammonium salts. The triphosphates were identified by characteristic ^{31}P NMR and by phosphorus analyses. Additional nucleotides used in the following studies were obtained commercially or as gifts.

Table I. Relative Affinities of Sugar-Modified GTP Analogues with p21[a]



compd	R	K_{rel}[b] EC	K_{rel}[b] Leu-61
GTP		1.10	1.20
dGTP		0.85	0.85
ddGTP		0.28	0.18
DHPGTP		0.0014	0.0018
ACVTP		0.0012	0.0011

[a] Compounds over a range of concentrations were allowed to compete with 3 μM [^{3}H]GDP in the presence of 1 μM p21. Conditions and workup of the assay are described in the Experimental Section. [b] K_{rel} is the ratio of concentration of [^{3}H]GDP to that of analogue which caused 50% inhibition of [^{3}H]GDP binding.

Results and Discussion

Relative Affinities of Sugar-Modified GTP Analogues to p21. A transforming *ras* oncogene of the Harvey (Ha) family commonly found in human cancers has a leucine codon replacing the glutamine codon for position 61 of the Ha-*ras* protein. Therefore, we chose to perform binding studies on the recombinant fusion proteins,[4] cellular Ha-*ras* protein, p21 EC, and the oncogenic form, p21 Leu-61. The assay for determining nucleotide affinities involved competition between [^{3}H]GDP and the test compounds for binding to the protein during incubation for 45 min at 37 °C, with the addition of EDTA to facilitate exchange of bound GDP.[23] After quenching exchange by the addition of excess Mg^{2+}, the mixtures were filtered through nitrocellulose disks, and the remaining [^{3}H]-GDP·p21 complex retained by the disks was counted. Relative binding affinities, K_{rel}, are defined as the ratio of the concentration of [^{3}H]GDP (3 μM) to that of analogue at which 50% inhibition of [^{3}H]GDP binding was observed.[23]

The effects of alterations in the sugar moiety of GTP, summarized in Table I, illustrate the minimal structural requirement for affinity to p21. Both GTP and its 2′-deoxy counterpart, dGTP, bound to the cellular (EC) and mutant (Leu-61) forms of p21 with similar affinities, as expected from the reported affinities of the two nucleotides for various forms of p21.[24] The removal of both sugar hy-

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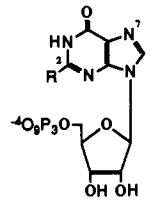
Table II. Relative Affinities of Base-Modified GTP Analogues with p21[a]



compd	R	K_{rel}[b] EC	Leu-61
GTP	$-NH_2$	1.10	1.20
ITP	-H	0.11	0.09
2-BrITP	-Br	0.04	0.05
7-DeazaGTP	$-NH_2$ (7=CH)	0.08	0.05
7-MeGTP	$-NH_2$ (7-Me)	0.0024	0.0020
1	-NH(*n*-Bu)	0.59	0.68
2	-NH(*p*-FC_6H_4)	1.77	2.38
3	-NH(*p*-BrC_6H_4)	2.28	2.10
4	-NH(*p*-tolyl)	1.14	1.34
6	-NH(*p*-*n*-BuC_6H_4)	3.30	6.00
5	-NH(4-PhBu)	1.19	1.10

[a] Compounds over a range of concentrations were allowed to compete with 3 μM [^{3}H]GDP in the presence of 1 μM p21. Conditions and workup of the assays are described in the Experimental Section. [b] K_{rel} is the ratio of concentration of [^{3}H]GDP to that of analogue which caused 50% inhibition of [^{3}H]GDP binding.

droxyls to give 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP) decreased affinity for both proteins by ca. 5-fold. The two acyclic nucleotides, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine triphosphate (DHPGTP) and 9-[(2-hydroxyethoxy)methyl]guanine triphosphate (ACVTP), showed drastically reduced binding to both forms of p21, with affinities ca. 3 orders of magnitude lower than those of GDP or GTP.

As observed in crystallographic structures, the glycosidic bond is anti and the ribose ring is C2'-endo in guanosine 5'-di- and triphosphate·p21 complexes.[12,13] A schematic representation of several relevant amino acids in the nucleotide binding site of p21, based on the complex with the nonhydrolyzable analogue guanosine 5'-(β,γ-imidotriphosphate) (GppNHp)[25] is shown in Figure 1, including distances in angstroms between heteroatoms involved in hydrogen bonds. A main chain carbonyl oxygen from valine (Val) 29 hydrogen bonds to the 2' hydroxyl group, and a water molecule hydrogen bonds to the 3'-hydroxyl group, of the ribose ring.[12,13] However, the lack of the 2'-hydroxyl group, in dGTP, does not appreciably reduce affinity to p21s, and 3'-O-substituted GTP derivatives were reported to bind well to p21s.[13] Loss of both 2'- and 3'-hydroxyls, in ddGTP, reduced affinities to p21 EC and p21 Leu-61 by only 4- and 6-fold, respectively. dGTP may be able to adopt a conformation to partially conserve hydrogen bonding of the 3'-hydroxyl group to Val-29, but ddGTP cannot provide a hydrogen-bond donor to the protein. The results of Table I do show that maintenance of the cyclic furanose structure is required for appreciable binding to p21. The acyclic nucleotides DHPGTP and ACVTP with flexible groups linking the base and triphosphate portions of the molecules displayed 3 orders of magnitude lower affinity to both proteins.

Relative Affinities of Base-Substituted GTP Analogues to p21. The effects of modifications in the guanine

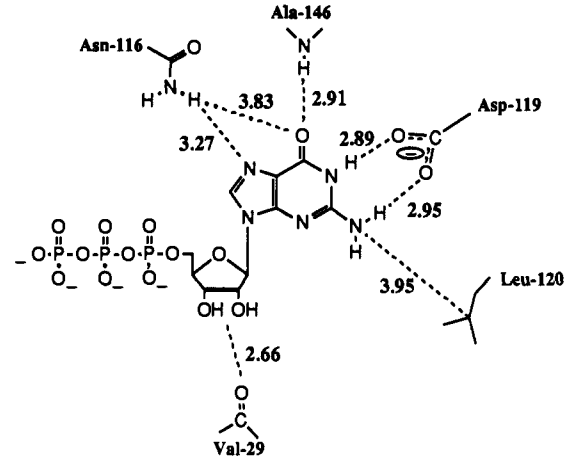


Figure 1. Schematic representation of selected protein–nucleotide interactions, based on X-ray crystal structure of the p21·GppNHp complex (ref 25). The dashed lines indicate specific interactions, and the corresponding distances are given in angstroms.

base of GTP on binding with EC and Leu-61 p21 are summarized in Table II. Inosine 5'-triphosphate (ITP) bound to both forms of p21 with affinities ca. 10-fold lower than those of GDP. 2-Bromoinosine 5'-triphosphate (2-BrITP), a compound containing a bromo group in place of the 2-amino group, displayed affinities ca. 20-fold lower than those of GDP. The decrease in affinity of these compounds for p21s, relative to GDP, emphasizes the importance of the exocyclic 2-amino group of GDP in binding with p21. Aspartate (Asp) 119 of p21 is part of the Asn-Lys-X-Asp motif (amino acids 116–119 in *ras* proteins) which is highly conserved in GTP binding proteins and appears to convey guanine nucleotide specificity to the proteins. X-ray crystallographic structures,[12,13] and the results of site-directed mutagenesis,[14] have shown that the carboxylate group of Asp-119 forms a cyclic hydrogen-bonded complex with a proton on the exocyclic 2-amino group and the proton on the endocyclic N1 of guanine (Figure 1). The reduction of affinity of ITP (Table II) is consistent with the loss of the hydrogen bond from the exocyclic amino proton, and the further reduction of affinity of 2-BrITP relative to ITP may result from repulsion between the bromo group and a carboxylate oxygen of Asp-119.

Removal of the 7-nitrogen of GTP resulted in decreased binding of nucleotide 7-deazaguanosine 5'-triphosphate (7-deazaGTP) to both EC and Leu-61 forms of p21 by 12- and 20-fold, respectively. Addition of a methyl group to N7, however, drastically reduced binding of the resulting 7-methylguanosine 5'-triphosphate (7-MeGTP) to both forms of p21. In the crystallographic structure of p21·GTP complexes,[13] it appears that the main chain NH of alanine (Ala) 146 forms a hydrogen bond to O6 of guanine, and the *syn*-hydrogen of the carboxamide group of asparagine (Asn) 116 forms a weak hydrogen bond with N7 (Figure 1). In fact, a similar distance between the *syn*-carboxamide hydrogen of Asn-116 and O6 of guanine suggests a cooperative arrangement of weak hydrogen bonds or electrostatic interactions between Asn-116 and N7 and O6 which may aid in the guanine specificity of p21. Perturbation of this arrangement may be responsible for the decreased binding of 7-deazaGTP to p21s, and is consistent with the results of site-directed mutagenesis. For example, the conservative substitution of glutamine for Asn-116 reduced affinities of the mutant p21s to GTP by about 10-fold,[26]

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(25) Pai, E. F.; Krengel, U.; Petsko, G. A.; Goody, R. S.; Kabsch, W.; Wittinghofer, A. *EMBO J.* **1990**, *9*, 2351–2359.
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similar in magnitude to the reductions observed for 7-deazaGTP (Table II). In the case of 7-methylGTP, steric and/or electronic affects may also contribute further to the drastic decrease in binding observed with this nucleotide. Indeed, the nonconservative replacement of isoleucine for Asn-116 gave mutant p21s without detectable binding to GTP.[26]

The results of Table II show that all GTP derivatives substituted on the exocyclic 2-amino group competed with GDP for both forms of p21, but to different extents. Alkyl derivative N^2-*n*-butylguanosine 5'-triphosphate (1) showed a 2-fold reduction in affinity for both EC and Leu-61 proteins, but all of the N^2-aryl-substituted derivatives showed equal or increased affinity for both forms of p21. N^2-(*p*-Fluorophenyl) derivative **2** had an affinity about 2-fold greater than GDP for both proteins, as did N^2-(*p*-bromophenyl)guanosine 5'-triphosphate, **3**. N^2-*p*-Tolyl and N^2-(4-phenylbutyl) derivatives **4** and **5** had affinities slightly higher and equal to that of GDP, respectively, for both proteins. N^2-[*p*-(*n*-Butyl)phenyl]guanosine 5'-triphosphate (**6**, BuPGTP), bound to EC p21 with an affinity 3-fold greater than that of GDP and to Leu-61 p21 with an affinity 6-fold greater than that of GDP. These results are consistent with X-ray crystal structures of p21s and the related GTP-binding protein EF-Tu,[27] which show that only one hydrogen bond is donated by the exocyclic 2-amino group of guanine (to Asp-119 of p21; see above), and indicate that substitution of the remaining hydrogen with large hydrophobic substituents is well-tolerated by the proteins. In the crystallographic structure of p21·GppNHp, this remaining hydrogen is projecting into an open area of the protein, a feature which may explain the tolerance for bulky substituents in this position of the ligand.

The basis for the enhanced binding of **6** to p21s is unclear, although there is a hydrophobic leucine residue (Leu-120) about 4 Å from the 2-amino nitrogen (Figure 1) which may interact with the N^2-substituents. An interaction between the side chain of Phe-28, which is situated above and perpendicular to the guanine ring, and the N^2-substituents is unlikely, because the closest distance between it and the 2-amino nitrogen of the nucleotide is 6 Å (not shown). Increased binding of **6** prompted us to test if the corresponding nucleoside or other phosphorylated forms would bind to p21s. As anticipated, the diphosphate (BuPGDP) was able to bind to both EC and Leu-61 with K_{rel} of 3.16 and 5.98, respectively, values similar to those of **6**. The monophosphate (BuPGMP) did bind to both EC and Leu-61 forms of p21, but with K_{rel} values of 0.0023 and 0.0027, respectively, 500-fold lower than the affinities of GDP. The nucleoside N^2-[*p*-(*n*-butyl)phenyl]guanosine, at 20 mM, did not bind to either EC or Leu-61 p21s.

Conclusions

The effects of structural changes in the guanine nucleotides on binding to cellular and oncogenic forms of Ha-*ras*-p21 presented in Tables I and II are consistent with X-ray crystallographic and site-directed mutagenesis results of the GTP/GDP site in this and related protein families. The interactions between amino acids and GTP depicted in Figure 1, based on the X-ray coordinates (1.35-Å resolution) of the p21·GppNHp complex[25] implemented on a Silicon Graphics computer system, highlight the interpretation of analogue binding reported in this paper. In contrast to the effects of opening of the ribose ring and blocking of sites crucial to interactions between nucleotide and protein, monosubstitution of the exocyclic 2-amino group of GTP did not interfere with the ability of the resulting nucleotides to bind to either form of p21.

The guanine specificity region of GTP binding proteins (Figure 1) consists of an Asp, such as that at position 119 in p21, which forms a cyclic hydrogen-bonded structure with the base, a main chain peptide NH (Ala-146 in p21) which hydrogen bonds to the guanine O6 atom, and an Asn (residue 116 in p21) which may form weak hydrogen bonds to guanine N7 and O6. (Asn-116 also serves, via a hydrogen bond between the *anti*-carboxamide hydrogen and the main chain carbonyl oxygen of Val-14,[25] to connect the phosphate binding loop of the protein to that of the base-binding region.) The Ala-146 NH and Asn-116 *syn*-carboxamide NH are above and below the plane of the guanine ring, respectively. Thus, a combination of weak interactions between the *syn*-carboxamide hydrogen of Asn-116 and both O6 and N7 of guanine, and the Ala-146 hydrogen bond to O6, may represent essential features for guanine specificity and affinity.

The results of this paper demonstrate that N^2-substituted GTPs can bind to normal and oncogenic forms of proteins of the *ras* family. Increased affinity of p21 for BuPGTP (**6**) could reflect interaction of the *p*-(*n*-butyl)-phenyl group with an amino acid side chain of the protein, such as that of Leu-120 (Figure 1). The difference in affinities of the cellular and oncogenic forms of p21 for this compound may be due to differences in the position of the interacting side chain in the proteins. Compounds such as **6** could, therefore, be used as probes to investigate structural differences between cellular and oncogenic forms of p21. Based on the X-ray crystallographic structures of p21·GDP/GTP complexes and the fact that BuPGMP had demonstrable affinity for p21 (see above), we are encouraged to search for analogues related to N^2-substituted guanines that may interact with specific amino acid side chains in p21 proteins and show affinity to selected p21 mutants in vitro and in vivo.

Experimental Section

The following compounds were synthesized as described previously: N^2-*p*-tolylguanosine (**4b**)[16] and N^2-[*p*-(*n*-butyl)-phenyl]guanosine 5'-triphosphate (**6**).[15] 9-[(2-Hydroxyethoxy)-methyl]guanine triphosphate (ACVTP) was a gift from Burroughs Wellcome Co. 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine triphosphate (DHPGTP) and the Ha-*ras* proteins[4] p21 EC and p21 Leu-61 were gifts from Merck, Sharp and Dohme Research Laboratories; 7-deazaGTP was a gift from Prof. F. Seela. Other nucleotides were obtained from Sigma, and [^{3}H]GDP (specific activity 10 Ci/mmol) was obtained from Amersham International Inc. Melting points were determined on a Mel-temp apparatus and are uncorrected. Analyses for C, H, N, and P were done by the Microanalysis Laboratory, University of Massachusetts, Amherst, MA; all analyses agreed to ±0.4% of the calculated values. NMR spectra were determined with a Bruker WM250 instrument; internal tetramethylsilane and an external capillary of 8.5% phosphoric acid served as references for ^{1}H and ^{31}P spectra, respectively. Only characteristic ^{1}H chemical shifts and coupling constants are given; all other resonances were as expected. UV spectra were obtained with a Gilford Response spectrophotometer. Molecular modeling employed a Silicon Graphics 70 GT computer system and the Quanta software supplied by Polygen Corp., Waltham, MA.

N^2-Substituted guanines were prepared from 2-bromohypoxanthine and the appropriate amines in refluxing 2-methoxyethanol as described.[16]

N^2-*n*-Butylguanine (**1a**) was obtained in 59% yield after crystallization from ethanol: mp 268–270 °C; ^{1}H NMR (Me_2SO-d_6) δ 12.40 (s, 9-H), 11.21 (s, 1-H), 7.66 (s, 8-H), 6.20 (s, 2-NH), 3.29 (m, CH_2N), 1.43 (m, $(CH_2)_2$), 0.94 (t, CH_3). Anal. ($C_9H_{13}N_5O \cdot {}^1/_6H_2O$) C, H, N.

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N^2-(*p*-Fluorophenyl)guanine (**2a**) was obtained in 63% yield after crystallization from ethanol: mp 350 °C; ^{1}H NMR (Me_2SO-d_6) δ 12.42 (s, 9-H), 10.51 (s, 1-H), 7.83 (s, 8-H), 7.45 (m, C_6H_4), 5.86 (s, 2-NH). Anal. ($C_{11}H_8N_5OF\cdot{}^1/_2H_2O$) C, H, N.

N^2-(*p*-Bromophenyl)guanine (**3a**) was obtained in 61% yield after crystallization from ethanol: mp 303–305 °C; ^{1}H NMR (Me_2SO-d_6) δ 12.75 (s, 9-H), 10.65 (s, 1-H), 9.19 (s, 2-NH), 7.74 (s, 8-H), 7.56 (m, C_6H_4). Anal. ($C_{11}H_8N_5OBr\cdot{}^1/_4H_2O$) C, H, N.

N^2-(4-Phenylbutyl)guanine (**5a**) was obtained in 81% yield after crystallization from ethanol: mp 237–239 °C; ^{1}H NMR (Me_2SO-d_6) δ 12.18 (s, 9-H), 10.31 (s, 1-H), 7.65 (s, 8-H), 7.21 (m, C_6H_5), 6.42 (s, 2-NH), 2.60 (m, CH_2N), 1.57 (m, $(CH_2)_3$). Anal. ($C_{15}H_{17}N_5O$) C, H, N.

N^2-Substituted-9-β-D-ribofuranosylguanines were prepared by glycosylation of the N^2-substituted guanines with tetra-*O*-acetylribose as described.[17,18] Reactions were followed by thin-layer chromatography (silica gel, 9:1 $CHCl_3/CH_3OH$), and the plates were developed with anisaldehyde spray. After ca. 6 h the reaction mixture was concentrated, and the resultant syrup was dissolved in chloroform. The chloroform layer was extracted with water until the pH of the aqueous phase was neutral, and the organic phase was evaporated to dryness. Crystallization of the acetylated products from ethanol gave the major isomer in each case. Each product was deblocked by treatment with methanol saturated with ammonia. Precipitated products were collected by filtration and crystallized from ethanol to give the following products.

N^2-*n*-Butyl-9-β-D-ribofuranosylguanine (**1b**) was isolated in 55% yield: mp 188–190 °C; ^{1}H NMR (Me_2SO-d_6) δ 7.92 (s, 8-H), 5.73 (d, J = 5.7 Hz, 1′-H), 4.55 (d, 2′-H), 4.13 (t, 3′-H), 3.90 (q, 4′-H), 3.60 (m, 5′,5″-H); UV (pH 10) λ_{max} 276 nm (ε 13020). Anal. ($C_{14}H_{21}N_5O_5\cdot H_2O$) C, H, N.

N^2-(*p*-Fluorophenyl)-9-β-D-ribofuranosylguanine (**2b**) was obtained in 49% yield: mp 266–269 °C; ^{1}H NMR (Me_2SO-d_6) δ 7.84 (s, 8-H), 5.71 (d, J = 5.4 Hz, 1′-H), 4.57 (d, 2′-H), 4.13 (t, 3′-H), 3.86 (q, 4′-H), 3.61 (m, 5′,5″-H); UV (pH 10) λ_{max} 272.5 nm (ε 13803). Anal. ($C_{16}H_{16}N_5O_5F\cdot{}^1/_3H_2O$) C, H, N.

N^2-(*p*-Bromophenyl)-9-β-D-ribofuranosylguanine (**3b**) was obtained in 52% yield: mp 208–210 °C; ^{1}H NMR (Me_2SO-d_6) δ 8.02 (s, 8-H), 5.70 (d, J = 6.0 Hz, 1′-H), 4.54 (d, 2′-H), 4.10 (t, 3′-H), 3.87 (q, 4′-H), 3.58 (m, 5′,5″-H); UV (pH 10) λ_{max} 282.5 nm (ε 13800). Anal. ($C_{16}H_{16}N_5O_5Br\cdot{}^1/_2H_2O$) C, H, N.

N^2-(4-Phenylbutyl)-9-β-D-ribofuranosylguanine (**5b**) was isolated in 62% yield: mp 230–235 °C; ^{1}H NMR (Me_2SO-d_6) δ 7.60 (s, 8-H), 5.68 (d, J = 5.4 Hz, 1′-H), 4.52 (d, 2′-H), 4.11 (t, 3′-H), 3.89 (q, 4′-H), 3.61 (m, 5′,5″-H); UV (pH 10) λ_{max} 285 (ε 12200), 255 nm (sh). Anal. ($C_{20}H_{25}N_5O_5$) C, H, N.

N^2-Substituted-9-β-D-ribofuranosylguanine 5′-phosphates were prepared by reaction of the nucleosides **1b–6b** with phosphoryl chloride in trimethyl phosphate at ca. −4 °C as described.[16] After 6 h an equal volume of water was added, and the pH of the solution was neutralized by the addition of 0.1 N NaOH. These solutions, in several portions, were purified by anion-exchange HPLC (see below), and the products were isolated by lyophilization as the ammonium salts.

N^2-*n*-Butyl-9-β-D-ribofuranosylguanine 5′-phosphate (**1c**) was obtained in 63% yield: ^{31}P NMR (D_2O) δ 3.92. Anal. ($C_{14}H_{17}N_5O_5P\cdot NH_4$) P.

N^2-(*p*-Fluorophenyl)-9-β-D-ribofuranosylguanine 5′-phosphate (**2c**) was obtained in 70% yield: ^{31}P NMR (D_2O) δ 3.92. Anal. ($C_{16}H_{17}N_5O_5FP\cdot 2NH_4$) P.

N^2-(*p*-Bromophenyl)-9-β-D-ribofuranosylguanine 5′-phosphate (**3c**) was obtained in 63% yield: ^{31}P NMR (D_2O) δ 3.88. Anal. ($C_{16}H_{17}N_5O_5BrP\cdot NH_4$) P.

N^2-*p*-Tolyl-9-β-D-ribofuranosylguanine 5′-phosphate (**4c**) was obtained in 64% yield: ^{31}P NMR (D_2O) δ 3.91. Anal. ($C_{17}H_{20}N_5O_5\cdot NH_4$) P.

N^2-(4-Phenylbutyl)-9-β-D-ribofuranosylguanine 5′-phosphate (**5c**) was obtained in 76% yield: ^{31}P NMR (D_2O) δ 3.51. Anal. ($C_{20}H_{25}N_5O_5P\cdot NH_4$) P.

2-Bromoinosine 5′-phosphate, prepared in the same manner from 2-bromoinosine,[18] was obtained in 69% yield: ^{31}P NMR (D_2O) δ 3.92. Anal. ($C_{10}H_9N_4O_8BrP\cdot NH_4$) P.

N^2-Substituted-9-β-D-ribofuranosylguanine 5′-triphosphates, 1–5, and 2-bromoinosine 5′-triphosphate were prepared by the condensation of the corresponding monophosphates, activated by 1,1′-carbonyldiimidazole, with tetra(tri-*n*-butylammonium) pyrophosphate in hexamethylphosphoramide as described.[16] After ca. 8 h the reaction mixtures were diluted with water, and the products were purified by anion-exchange HPLC (see below) and isolated after lyophilization as the ammonium salts.

N^2-*n*-Butyl-9-β-D-ribofuranosylguanine 5′-triphosphate (**1**) was obtained in 46% yield: ^{31}P NMR (D_2O) δ −6.10 (P_γ, d, $J_{\beta,\gamma}$ = 20.2 Hz), −11.22 (P_α, dt, $J_{\alpha,\beta}$ = 20.2 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.4 Hz), −22.14 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 20.2 Hz). Anal. ($C_{14}H_{21}N_5O_{14}P_3\cdot 3NH_4$) P.

N^2-(*p*-Fluorophenyl)-9-β-D-ribofuranosylguanine 5′-triphosphate (**2**) was obtained in 57% yield: ^{31}P NMR (D_2O) δ −5.79 (P_γ, d, $J_{\beta,\gamma}$ = 19.8 Hz), −11.02 (P_α, dt, $J_{\alpha,\beta}$ = 19.8 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.7 Hz), −22.92 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 19.8 Hz). Anal. ($C_{16}H_{17}N_5O_{14}FP_3\cdot 3NH_4$) P.

N^2-(*p*-Bromophenyl)-9-β-D-ribofuranosylguanine 5′-triphosphate (**3**) was obtained in 49% yield: ^{31}P NMR (D_2O) δ −7.48 (P_γ, d, $J_{\beta,\gamma}$ = 19.4 Hz), −10.90 (P_α, dt, $J_{\alpha,\beta}$ = 19.4 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.7 Hz), −22.02 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 19.4 Hz). Anal. ($C_{16}H_{17}N_5O_{14}BrP_3\cdot 3NH_4$) P.

N^2-*p*-Tolyl-9-β-D-ribofuranosylguanine 5′-triphosphate (**4**) was obtained in 50% yield: ^{31}P NMR (D_2O) δ −5.88 (P_γ, d, $J_{\beta,\gamma}$ = 19.8 Hz), −10.93 (P_α, dt, $J_{\alpha,\beta}$ = 19.8 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.6 Hz), −21.62 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 19.8 Hz). Anal. ($C_{17}H_{20}N_5O_{14}P_3\cdot 3NH_4$) P.

N^2-(4-Phenylbutyl)-9-β-D-ribofuranosylguanine 5′-triphosphate (**5**) was obtained in 35% yield: ^{31}P NMR (D_2O) δ −5.45 (P_γ, d, $J_{\beta,\gamma}$ = 19.6 Hz), −11.06 (P_α, dt, $J_{\alpha,\beta}$ = 19.6 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.6 Hz), −22.94 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 19.6 Hz). Anal. ($C_{20}H_{25}N_5O_{14}P_3\cdot 3NH_4$) P.

2-Bromoinosine 5′-triphosphate was obtained in 35% yield: ^{31}P NMR (D_2O) δ −5.45 (P_γ, d, $J_{\beta,\gamma}$ = 20.2 Hz), −10.34 (P_α, dt, $J_{\alpha,\beta}$ = 20.2 Hz, $J_{\alpha,5',5''\cdot H}$ = 4.6 Hz), −21.41 (P_β, t, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 20.2 Hz). Anal. ($C_{10}H_9N_4O_{14}BrP_3\cdot 3NH_4$) P.

High-Performance Liquid Chromatography. A semipreparative anion-exchange HPLC method employed the following components: Waters Associates 510 pump, ISCO 2360 gradient programmer, and Knauer UV photometer detector. A Synchropak AX-100 column (250 × 10 mm) was used under the following conditions: injection volume, 2 mL; detection, UV 254 nm; temperature, ambient; eluents, buffer A (35% acetonitrile) and buffer B (0.5 M ammonium bicarbonate, pH ~7.4, 35% acetonitrile). Nucleotides were purified under these conditions with a gradient of 100% buffer A to 100% buffer B during 30 min. The flow rate was normally 8.5 mL/min, and the peaks corresponding to the products were collected and lyophilized directly.

Nucleotide Binding Assay. Affinity of the analogues for the Ha-*ras* proteins, p21, was determined by a filter binding assay adapted from those of Gibbs et al.[4] and Tucker et al.[22] One microgram of p21 (as the complex with GDP) was incubated at 37 °C for 45 min in 50 μL of a mixture containing 50 mM Tris·HCl (pH 7.5), 10 mM dithiothreitol, 0.5 mM $MgCl_2$, 100 mM NH_4Cl, 5 mM EDTA, and 2 μM [^{3}H]GDP (specific activity 10 Ci/mmol). Appropriate amounts of test nucleotides dissolved in water or an equal volume of water alone were present during the assay period. Exchange reactions were quenched by the addition of 1.5 mL of a cold solution of 1 mM $MgCl_2$ in 50 mM Tris·HCl (pH 6.8). The solutions were filtered through nitrocellulose disks (BA85, Schleicher and Schuell), and the disks were washed with quenching solution (2 × 1.5 mL). The disks were dried, and the filter-bound radioactivity was determined by scintillation counting of each disk in 5 mL of Omnifluor (New England Nuclear). Control binding to p21 typically was calculated to be 0.8–0.9 pmol of GDP/pmol of p21.

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Registry No. 1·3NH_3, 131933-74-9; **1a**, 114300-69-5; **1b**, 131933-83-0; **1c**·NH_3, 131933-87-4; **2**·3NH_3, 131933-75-0; **2a**,

131933-80-7; **2b**, 131933-84-1; **2c**·2NH_3, 131933-88-5; **3**·3NH_3, 131933-76-1; **3a**, 123994-72-9; **3b**, 131933-85-2; **3c**·NH_3, 131933-89-6; **4**·3NH_3, 131933-77-2; **4b**, 77976-95-5; **4c**·NH_3, 131933-90-9; **5**·3NH_3, 131933-78-3; **5a**, 131933-81-8; **5b**, 131933-86-3; **5c**·NH_3, 131933-91-0; **6**·3NH_3, 131933-79-4; dGTP·3NH_3, 131933-93-2; ddGTP·NH_3, 132072-09-4; DHPGTP·3NH_3, 131933-94-3; ACVTP·3NH_3, 131933-95-4; ITP·3NH_3, 131933-96-5; 2-BrITP·3NH_3, 131933-97-6; 7-deazaGTP·3NH_3, 131933-98-7; 7-MeGTP, 26554-26-7; 2-bromohypoxanthine, 87781-93-9; 2-bromoinosine, 131933-82-9.

Synthesis and Biochemical Evaluation of Baclofen Analogues Locked in the Baclofen Solid-State Conformation

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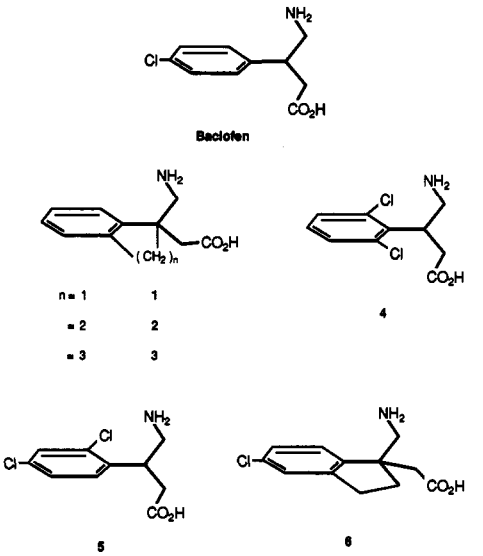
The synthesis of six close analogues of baclofen [3-(4-chlorophenyl)-4-aminobutyric acid] (BAC), a potent $GABA_B$ agonist, are reported. The compounds were designed starting from the structural informations contained in the solid state of BAC, regarded as a possible bioactive conformation, in which the *p*-chlorophenyl ring is perpendicular to the GABA backbone. A similar conformational situation was created by rigidifying the BAC structure by means of methylene (**1**), ethylene (**2** and **6**), or propylene (**3**) units, or by introducing chlorine atoms (**4** and **5**) into the ortho positions ("ortho effect"). Only compound **5** showed affinity for the $GABA_B$ receptor. Compound **6** [1-(aminomethyl)-5-chloro-2,3-dihydro-1*H*-indene-1-acetic acid], which was initially considered as representing the optimal mimic of the solid-state conformation of BAC, was surprisingly found inactive. An extensive conformational analysis was performed on compounds **1–6** in order to evaluate their flexibility and the overlap of their conformational population with respect to BAC. For this purpose a distance map was generated from three possible pharmacophoric groups: the amino and the carboxylic functions, and the phenyl ring. Finally, several explanations are proposed to account for the poor affinities of the prepared compounds such as steric hindrance or flexibility demand of the receptor.

Introduction

For the inhibitory neurotransmitter γ-aminobutyric acid (GABA) two major receptor subtypes ($GABA_A$ and $GABA_B$) have been identified on the basis of electrophysiological[1,2] and binding studies.[3] There are evidences that these two receptors play an important part in the central and peripheral nervous system through ion-channel regulation.[4] The overall physiological effects are transmission inhibitions mediated pre- and post-synaptically by the $GABA_A$ sites and presynaptically by the $GABA_B$ sites.[5] A number of specific agonists or antagonists at the $GABA_A$ receptor site have been developed during the last decade.[6] In contrast, 3-(4-chlorophenyl)-4-aminobutyric acid (baclofen, BAC) is the only potent and selective $GABA_B$ agonist described until now. Among the reported $GABA_B$ antagonists such as 5-aminovaleric acid[7] or 3-aminopropanesulfonic acid[8] none has the required potency or selectivity. Phaclofen [3-amino-2-(4-chlorophenyl)-propylphosphonic acid][9] and 2-hydroxysaclofen [3-amino-2-(4-chlorophenyl)-2-hydroxypropanesulfonic acid][10] have been recently presented as peripheral and central BAC antagonists. Finally, the phosphonous analogue of GABA (3-aminopropylphosphinic acid) is reported as a potent ligand for the $GABA_B$ receptors.[11] But the parsimony of the literature data in the $GABA_B$ field does not allow a rational design of new $GABA_B$ ligands.[5,12]

According to recent work, a coupling between $GABA_B$ receptors and GTP binding[13,14] which mediates adenylate cyclase inhibition has been reported; moreover $GABA_B$ receptors modulate the liberation of neurotransmitters via calcium conductance.[15,16] Therefore it seemed worth our searching for new $GABA_B$ agonists or antagonists in order to gain additional knowledge of the $GABA_B$ receptors.



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