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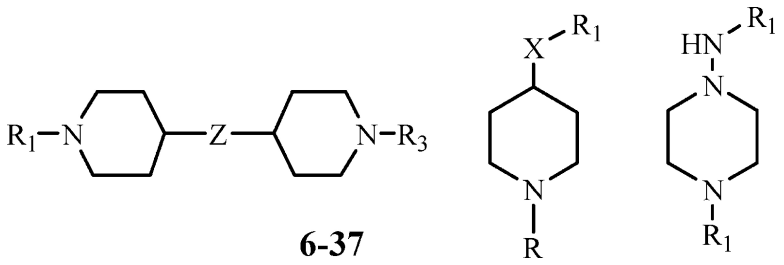

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Design, Synthesis, and Preliminary Pharmacological Evaluation of a Set of Small Molecules That Directly Activate Gi Proteins

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Heterotrimeric G proteins play a pivotal role in the communication of cells with the environment. G proteins are stimulated by cell surface receptors (GPCR) that catalyze the exchange of GDP, bound to Gα subunit, with GTP and can per se be the target of drugs. Based on the structure of two nonpeptidic modulators of Gi proteins, a series of new molecules characterized by a long hydrophobic chain and at least two nitrogen atoms protonated at physiological pH was designed. The compounds were tested for their ability to stimulate binding of GTPγS to recombinant Gi proteins. Gi activation properties were also evaluated by inhibition of adenylyl cyclase activity in intact lymphocytes. Most compounds were able to stimulate GTPγS binding and to inhibit cAMP production at micromolar doses. Among the active compounds, **34** showed good efficacy and was the most potent compound studied, particularly on α_o subtype; its regioisomer, **36**, was the most efficacious one. Compound **7** showed also an interesting profile as it showed selectivity toward the α_o subtype, in both efficacy and potency. Some of the compounds synthesized and found to be active may be useful leads to develop more potent and selective Gi protein modulators.

Introduction

Heterotrimeric G proteins play a pivotal role in the communication of cells with the environment. G proteins are stimulated by cell surface receptors [G protein-coupled receptors (GPCR)] that catalyze the exchange of GDP, bound to Gα subunit, with GTP. Consequently, the GTP-bound G protein dissociates into two signaling entities: the Gα subunit and the G$\beta\gamma$ subunit complex; β and γ subunits form tight complexes that cannot be dissociated under non-denaturing conditions.[1]

Gα and G$\beta\gamma$ both modulate cellular effectors. Today, several subtypes of Gα subunits are known, and stimulatory or inhibitory families can be identified. Inactivation occurs by the intrinsic GTPase activity of the Gα subunit; GDP-bound Gα subunit reassociates with G$\beta\gamma$ subunits, and this causes mutual inactivation because the effector's binding surfaces are inaccessible in the oligomer.

In this cycle of activation and deactivation, specific binding sites on G proteins allow for the sequential, conformation-dependent binding of protein reaction partners. These include receptors that interact with all three subunits, effectors, and regulators of G protein signaling (RGS), which bind to the transition state of Gα−GTP and exert a GTPase activating effect. The increased GTP turnover not only accelerates the rate of signal deactivation but also enhances the rate of activation.[2,3] In addition, several modulators have been identified that activate G protein signaling in the absence of a GPCR and that cannot be readily placed into this basal reaction cycle. These include activators of G protein signaling such as AGS1 peptide that apparently promotes nucleotide exchange on Gi proteins,[4] PCP2, a nucleotide exchange factor for $G\alpha_o$,[5] AGS2 that, in protein interaction studies, selectively associates with G$\beta\gamma$,[6,7] and AGS3[7] that interacts with Gα−GDP, preventing the interaction of Gα−GDP with G$\beta\gamma$; alternatively, it has been proposed that AGS3 may simply compete with G$\beta\gamma$ for binding to the Gα subunit.[8] Thus, several specific binding sites exist on the Gα subunit that may be exploited for the design of synthetic stimulatory or inhibitory compounds. In both experimental pharmacology and clinical pharmacotherapy, G protein-dependent signaling pathways are activated or inhibited by employing appropriate receptor agonists or antagonists, respectively. Several arguments indicate that G proteins can per se also be targeted by drugs and that this approach may, at least conceptually, offer advantages.[1] One of the most convincing arguments in favor of direct G protein targeting is the known promiscuity of receptors that often are able to couple with different classes of G proteins.[9] Thus, cellular stimulation by a receptor may result in the concerted activation of several distinct G proteins driving the activation of multiple effectors pathways. In principle, it might be useful to block signaling of a receptor via one type of G protein but not via the other type, a goal that cannot

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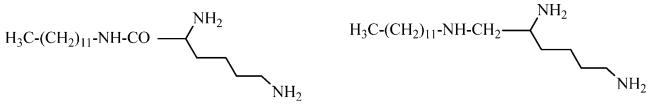
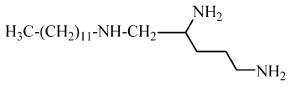
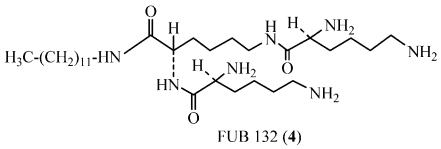
Chart 1



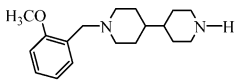
N-dodecylysinamide (**1**) and reduced derivative (**2**)



FUB 86 (**3**)



FUB 132 (**4**)



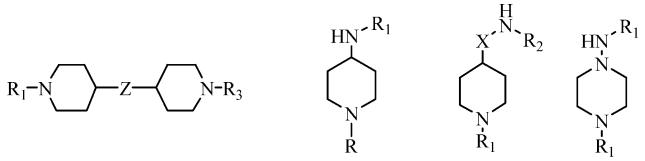
ML 202 (**5**)

be achieved with receptor antagonists but that may be possible with direct interaction with the individual G protein.[10] Some low molecular weight compounds have been identified that bind directly to G proteins.[11−13] Suramin and analogues such as NF 023 have been reported to act as nonselective G protein inactivators.[10,14,15] Mechanistic aspects have been studied in detail for receptor-derived peptides[16] and the receptor-mimetic peptide Mastoparan.[17,18]

Mastoparan (a 14 aa peptide from wasp venom), by mimicking the structure of activated receptor, selectively stimulates inhibitory Gα subunits ($G\alpha_{i/o}$). On the other hand, nonpeptidic modulators of G proteins are rare. As a matter of fact, because these molecules have to penetrate the membrane, their pharmacokinetic properties must be quite stringent. Some amphiphilic β-adrenoreceptor antagonists such as propranolol and local anesthetics such as bupivacain have been reported to be direct activators of Gi proteins, but at fairly high doses,[19] and the same has been claimed about some taste substances such as quinine and aspartame.[20] In 1997 Leschke and co-workers[21] described alkyl-substituted amino acid amides and analogues that act as new synthetic nonpeptidic direct $G\alpha_{i/o}$ protein activators such as *N*-dodecyl lysinamide (**1**), its reduced derivative (**2**), and FUB 86 (**3**) (Chart 1); later, a more efficient compound, FUB 132 (**4**), was developed.[22] More recently, Melchiorre and co-workers[23] reported the protein Gi activating properties of several molecules including compound **5** (Chart 1).

Nearly all inhibitory neurotransmitters able to enhance the pain threshold utilize Gi proteins as signal transduction system. Gi proteins represent the most widespread modulatory signaling pathway in neurones,[24] and their involvement in the modulation of pain perception has been well established. The administration of pertussis toxin (PTX), which selectively inactivates Gi proteins, produced hyperalgesia and allodynia in laboratory animals.[25,26] An increase of pain perception has also been observed in knock-out mice that lack the gene coding the subtype Go of the Gi protein family.[27]

Chart 2



R = H, CH_2-CH_2-NH_2, $C_{15}H_{31}$, $COC_{14}H_{29}$
R_1 = H, $COC_{12}H_{25}$, $COC_{14}H_{29}$, $COC_{16}H_{33}$, $C_{13}H_{27}$, $C_{15}H_{31}$, $C_{17}H_{35}$
R_2 = $C_{15}H_{31}$
R_3 = H, CH_3
Z = CH_2CH_2, NH, none
X = CO, CH_2

These data clearly indicate that a lack of functionality of Gi proteins enhances the sensitivity to pain.

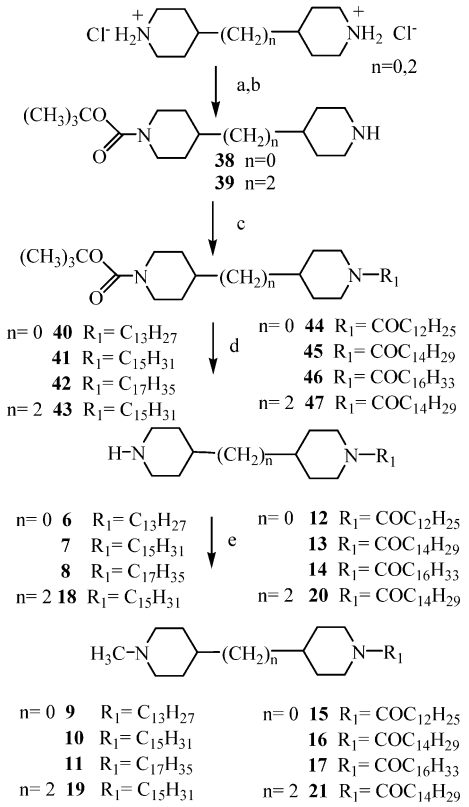
Hypofunctionality of Gi proteins also produced insensitivity to analgesic treatments acting via Gi stimulation. Thus, it has been observed that PTX prevents the enhancement of pain threshold induced by a wide variety of drugs with analgesic properties such as opioids, α_2-agonists, tricyclic antidepressants, and antihistamines.[25,28,29] These results are also supported by antisense studies in which the inhibition of the expression of the $G\alpha_i$ protein subunits prevented the analgesia induced by antihistaminic drugs and tricyclic antidepressants.[30,31] A selective involvement of specific Gi protein subtypes in the mechanism of action of analgesic compounds has also been demonstrated. The administration of selective antibodies against $G\alpha_{i/2}$, as well as the inhibition of its expression by the use of specific antisense oligonucleotides, prevented the analgesia induced by agonists of μ-opioid receptors.[32,33] The use of an antisense oligonucleotide (aODN) against $G\alpha_{i/3}$ demonstrated the essential role played by the $G\alpha_{i/3}$ subtype in increasing the pain threshold induced by α_2-adrenoceptor agonists.[34]

In 2001, some of us reported that hypofunctionality of Gi proteins is the etiopathogenic mechanism for migraine and cluster headaches.[35] This discovery has of course raised interest in small molecules able to restore Gi functionality, bypassing the receptors implied, to treat this pathological state, and we have started research to identify molecules directly activating Gi proteins. In this respect it is interesting that most major analgesics (morphine, baclofen, clonidine) are known to act through receptors that induce Gi protein activation.[25] Thus, in theory, identification of selective and potent Gi activators could result in the discovery of potent "universal" analgesics, which act independently from receptor activation. Toward this end, we have designed a series of new molecules that combine the characteristics of compounds **1**, **2**, and **5**. These new structures (Chart 2) present some of the features of molecules able to interact with Gi proteins that apparently are (i) a long hydrophobic chain (which need not be peptidic); (ii) possession of at least two positive charges.[36]

Chemistry

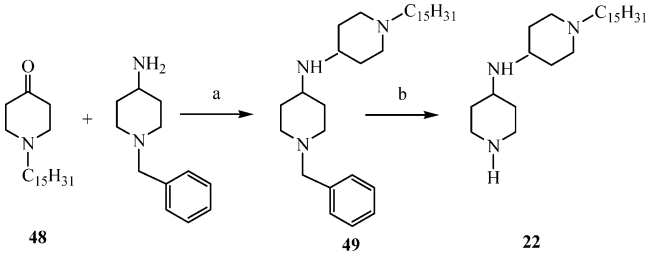
4,4′-Bipiperidines and 4,4′-Ethylenedipiperidines. Compounds **6**−**21** were synthesized as shown in Scheme 1. Commercially available 4,4′-bipiperidine dihydrochloride or 4,4′-ethylenedipiperidine dihydrochloride was

Scheme 1[a]



[a] Reaction conditions: (a) 10% NaOH; (b) BOC-ON; (c) bromoalkane or alkanoyl chloride, NEt_3; (d) 6 M HCl; (e) HCOOH, HCHO.

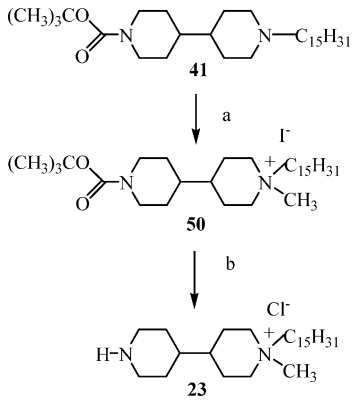
Scheme 2[a]



[a] Reaction conditions: (a) $(iPrO)_4Ti$, $NaBH_3CN$; (b) H_2, 10% Pd/C.

treated with 10% NaOH to obtain the free base and then reacted with BOC-ON [2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile][23] to give derivatives **38** and **39**. These compounds were treated with bromoalkyl derivatives and NEt_3 as a scavenger, to give **40**−**43**, or with the proper alkanoyl chloride, obtained from the acid with standard method,[37] to give **44**−**47**. Compounds **40**−**47** were then deprotected with 6 M HCl[23] to give **6**−**8**, **12**−**14**, **18**, and **20**. Finally, these compounds were treated with formic acid and formaldehyde[38] to give the corresponding methyl derivatives **9**−**11**, **15**−**17**, **19**, and **21**. Compound **22** has been synthesized as reported in Scheme 2. 1-Pentadecyl-piperidin-4-one **48**, obtained from 4-piperidone monohydrate hydrochloride and bromopentadecane through a synthetic pathway different from that reported in the literature,[39] was treated, under reductive conditions,[40] with 4-benzyl-1-amino piperidine to give **49**. Compound **49** was finally catalytically hydrogenated to obtain **22**. As shown in Scheme 3, **41**, treated with an excess of CH_3I in

Scheme 3[a]



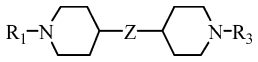
[a] Reaction conditions: (a) CH_3I, Et_2O; (b) 6 M HCl.

anhydrous ether, gave the salt **50**, which was then deprotected with 6 M HCl[23] to afford the final compound **23**. Chemical and physical characteristics of compounds **6**−**22** are reported in Table 1.

Piperidines. In Scheme 4 is shown the synthetic pathway used to obtain derivatives **24**−**27**. Commercially available 4-amino-1-benzylpiperidine, treated with the proper alkyl bromide or with pentadecanoyl chloride and NEt_3, afforded alkyl derivatives **51**−**53** and the acyl derivative **54**, which were catalytically hydrogenated to give the final products **24**−**27**. As reported in Scheme 5, *N*-benzyl isonipecotic acid ethyl ester **55**,[41] obtained from ethyl isonipecotate and benzyl chloride,[42] was hydrolyzed with NaOH to give *N*-benzyl isonipecotic acid **56**. The acid was then transformed into the corresponding amide **57** using ethyl chloroformate, NEt_3, and pentadecylamine[43] and hydrogenated to give **30**. Alkylation of **30** with bromoethylamine hydrobromide gave **31a** and **31b**; **31a**, after reduction with borane−methyl sulfide complex,[44] afforded compound **33**. The same reduction reaction afforded intermediate **58** from **57**. Finally, compound **32** was obtained by catalytic hydrogenation of **58**. According to Scheme 6, 1-pentadecyl-4-piperidone **48** was then transformed into **28** by reductive alkylation.[45] Compound **29** was obtained through two synthetic pathways that present similar yields despite the different number of synthetic steps. In the first attempt, 1-pentadecanoyl-4-piperidone **59**, obtained from 4-piperidone hydrochloride monohydrate with pentadecanoyl chloride, was transformed by reductive alkylation[40] into **60** that, in turn, was hydrogenated to **29** with 10% Pd/C. The same compound was more efficiently obtained from **59** by reductive alkylation with ammonium acetate.[45] Chemical and physical characteristics of compounds **24**−**29** and **30**−**33** are reported in Tables 2 and 3, respectively.

Piperazines. Compounds **34**−**37** were synthesized as shown in Scheme 7. Mononitrosopiperazine[46] was treated with bromopentadecane or pentadecanoyl chloride to give **61** and **62**, which were then reduced with Zn and HCl[46] to give **34** and **35**. Reduction with Zn and HCl of 4-benzyl-1-nitrosopiperazine gave 1-amino-4-benzylpiperazine **63**,[46] which was treated with bromopentadecane or pentadecanoyl chloride to give **64** and **65**, the hydrogenation of which with $HCOONH_4$ and 10% Pd/C in MeOH gave **36** and **37**, respectively. Chemical and physical characteristics of compounds **34**−**37** are reported in Table 4.

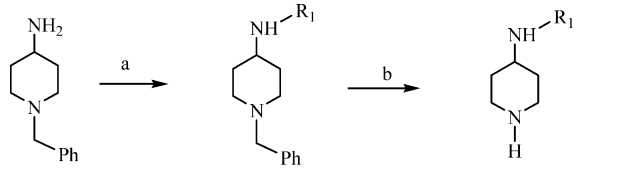
Table 1



no.	Z	R_3	R_1	mp (°C) base	salt	mp (°C) salt	analysis
6		H	$CH_2-C_{12}H_{25}$	61−62	2HCl	>270	$C_{23}H_{48}Cl_2N_2$
7		H	$CH_2-C_{14}H_{29}$	54−55	2HCl	>290	$C_{25}H_{52}Cl_2N_2$
8		H	$CH_2-C_{16}H_{33}$	56−57	2HCl	>270	$C_{27}H_{56}Cl_2N_2$
9		CH_3	$CH_2-C_{12}H_{25}$	<30	2HCl	>270	$C_{24}H_{50}Cl_2N_2$
10		CH_3	$CH_2-C_{14}H_{29}$	30−31[a]	2HCl	>270	$C_{26}H_{54}Cl_2N_2$
11		CH_3	$CH_2-C_{16}H_{33}$	30−31[b]	2HCl	>270	$C_{28}H_{58}Cl_2N_2$
12		H	$CO-C_{12}H_{25}$	56−58	HCl	150−153	$C_{23}H_{45}ClN_2O$
13		H	$CO-C_{14}H_{29}$	43−44	HCl	167−168	$C_{25}H_{49}ClN_2O$
14		H	$CO-C_{16}H_{33}$	43−45	HCl	165−167	$C_{27}H_{53}ClN_2O$
15		CH_3	$CO-C_{12}H_{25}$	<30	HCl	140−143	$C_{24}H_{47}ClN_2O$
16		CH_3	$CO-C_{14}H_{29}$	<30	HCl	167−169	$C_{26}H_{51}ClN_2O$
17		CH_3	$CO-C_{16}H_{33}$	<30	HCl	168−170	$C_{28}H_{55}ClN_2O$
18	CH_2CH_2	H	$CH_2-C_{14}H_{29}$	48−50	2HCl	250−253	$C_{27}H_{56}Cl_2N_2$
19	CH_2CH_2	CH_3	$CH_2-C_{14}H_{29}$	53−54	2HCl	>270	$C_{28}H_{58}Cl_2N_2$
20	CH_2CH_2	H	$CO-C_{14}H_{29}$	30−32	HCl	162−163	$C_{27}H_{53}ClN_2O$
21	CH_2CH_2	CH_3	$CO-C_{14}H_{29}$	42−43	HCl	95−97	$C_{28}H_{55}ClN_2O$
22	NH	H	$CH_2-C_{14}H_{29}$	200−202	3HCl	>270	$C_{25}H_{54}Cl_3N_3$

[a] Compound has been purified by column chromatography using eluent B. [b] Compound has been purified by column chromatography using eluent C (see Experimental Section).

Scheme 4[a]

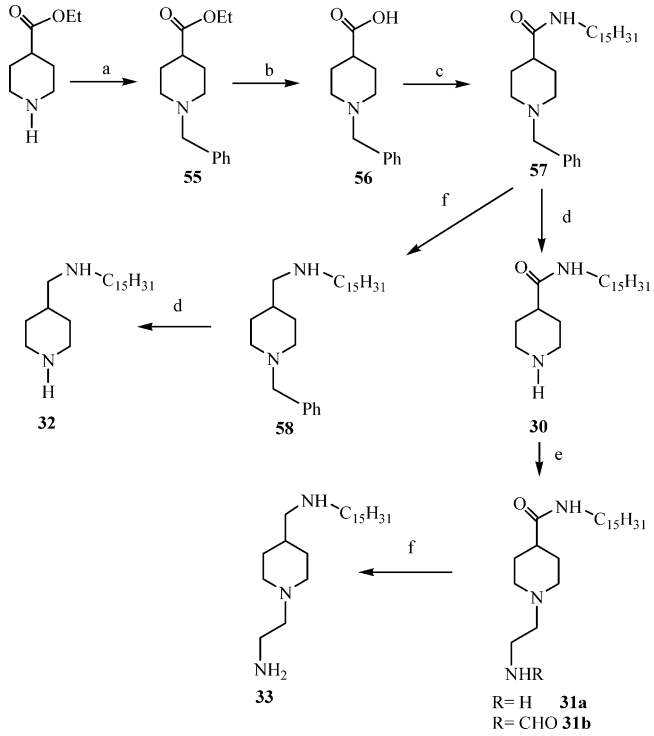


51 $R_1 = C_{13}H_{27}$
52 $R_1 = C_{15}H_{31}$
53 $R_1 = C_{17}H_{35}$
54 $R_1 = COC_{14}H_{29}$

24 $R_1 = C_{13}H_{27}$
25 $R_1 = C_{15}H_{31}$
26 $R_1 = C_{17}H_{35}$
27 $R_1 = COC_{14}H_{29}$

[a] Reaction conditions: (a) alkyl bromide or pentadecanoyl chloride, NEt_3; (b) H_2, 10% Pd/C.

Scheme 5[a]



[a] Reaction conditions: (a) benzyl chloride, NEt_3; (b) NaOH; (c) EtOCOCl, NEt_3, pentadecylamine; (d) H_2, 10% Pd/C; (e) bromoethylamine; (f) $(CH_3)_2S{\cdot}BH_3$.

Pharmacology

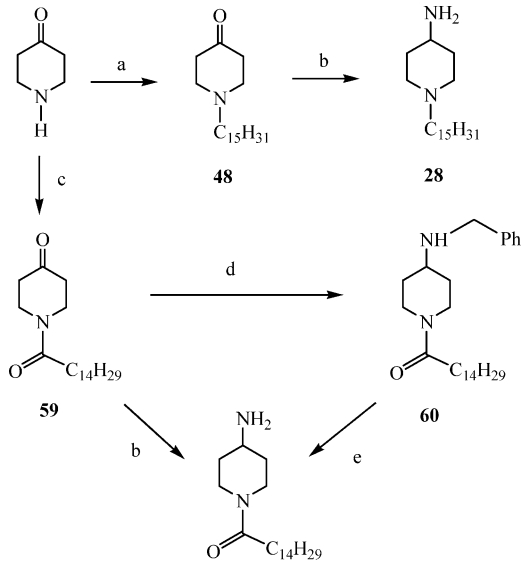
The compounds were tested for their ability to stimulate binding of GTPγS to recombinant Gi proteins, which was taken as a measure of their direct interaction with Gi proteins. Moreover, to check their Gi-activating properties we evaluated the inhibition of adenylyl cyclase activity in intact lymphocytes. Although not specific, this assay would provide information on their ability to cross the cell membrane, a critical requisite for the kind of activity we were seeking.

GTPγS(^{35}S) Binding to Recombinant Gi Proteins. Gi proteins are coupled to many receptor types and subtypes. It is also well-known that Gi proteins can be down-regulated to several levels, such as turnover and transcription, by hormones and neurotransmitters. To exclude the investigated compounds from exerting their activity in a receptor-mediated manner, the ability of the compounds to directly activate Gi proteins was evaluated on recombinant Gi isoforms. Recombinant $G\alpha_{i/o}$ subunits were reconstituted in an in vitro system, and GTPγS binding was measured to verify the protein activation level in the presence of the tested compounds. To unmask a potential selectivity of these molecules toward Gi protein isoforms, α_{i1}, α_{i3}, and α_{o1} subunits were expressed and purified. Binding of GTPγS (the nonhydrolizable GTP analogue) was then measured in the presence of our compounds that, if endowed with Gi protein stimulatory activity, would increase the basal binding of GTPγS. **1** and **5** were used as reference compounds.

Gi Protein Modulation. Inhibition of adenylyl cyclase activity in intact lymphocytes from human peripheral blood was measured, taking into account that Gi proteins have a ubiquitous distribution.[47] Lymphocytes represent an appropriate tool to detect Gi protein functionality because they have cell surface recognition sites for pertussis toxin, the selective inactivator of Gi. These recognition sites are lacking in other blood elements such as erythrocytes.[48]

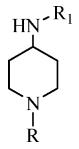
Enzymatic activity was previously amplified by forskolin, a diterpene that selectively activates adenylyl

Scheme 6[a]



[a] Reaction conditions: (a) bromopentadecane, NEt_3; (b) CH_3COONH_4, $NaBH_3CN$; (c) pentadecanoyl chloride, NEt_3; (d) benzylamine, $(iPrO)_4Ti$, $NaBH_3CN$; (e) H_2, 10% Pd/C.

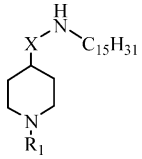
Table 2



no.	R	R_1	mp (°C) base	salt	mp (°C) salt	analysis
24	H	$CH_2-C_{12}H_{25}$	30−31[a]	2HCl	268−269	$C_{18}H_{40}Cl_2N_2$
25	H	$CH_2-C_{14}H_{29}$	42−43	2HCl	245−246	$C_{20}H_{44}Cl_2N_2$
26	H	$CH_2-C_{16}H_{33}$	30−32[b]	2HCl	250−251	$C_{22}H_{48}Cl_2N_2$
27	H	$CO-C_{14}H_{29}$		HCl	106−108	$C_{20}H_{41}ClN_2O$
28	$CH_2-C_{14}H_{29}$	H	*a*	2HCl	>240	$C_{20}H_{44}Cl_2N_2$
29	$CO-C_{14}H_{29}$	H	50−51[a]	HCl	210−212	$C_{20}H_{41}ClN_2O$

[a] Compound has been purified by column chromatography using eluent B. [b] Compound has been purified by column chromatography using eluent D (see Experimental Section).

Table 3

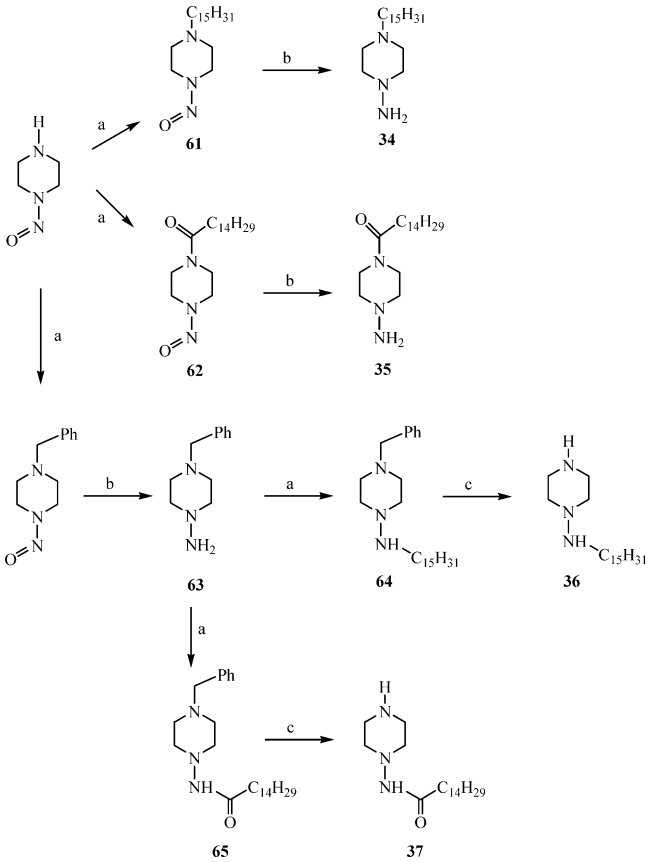


no.	R_1	X	mp (°C) base	salt	mp (°C) salt	analysis
30	H	CO	73−74	HCl	220−221	$C_{21}H_{43}ClN_2O$
31a	$CH_2CH_2NH_2$	CO	100−101	2HCl	218−220	$C_{23}H_{49}Cl_2N_3O$
31b	CH_2CH_2NHCHO	CO	109−110	HCl	201−203	$C_{24}H_{48}ClN_3O_2$
32	H	CH_2	31−33	2HCl	222−225	$C_{21}H_{46}Cl_2N_2$
33	$CH_2CH_2NH_2$	CH_2	40−43[a]	3HCl	215−220	$C_{23}H_{52}Cl_3N_3$

[a] Compound has been purified by column chromatography using eluent D (see Experimental Section).

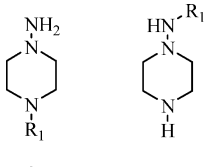
cyclase,[49] at a concentration of 10^{-4} M, which is reported to be the most effective one in human lymphocytes.[50] The forskolin-stimulated adenylyl cyclase activity was inhibited, with similar potency, by the nonhydrolizable GTP analogue Gpp(NH)p, used as physiological control,

Scheme 7[a]



[a] Reaction conditions: (a) alkyl bromide or alkanoyl chloride, NEt_3; (b) Zn, HCl; (c) $HCOONH_4$, 10% Pd/C, MeOH.

Table 4



no.	structure	R_1	mp (°C) base	salt	mp (°C) salt	analysis
34	A	$CH_2-C_{14}H_{29}$	30−32[a]	3HCl	217−218	$C_{19}H_{44}Cl_3N_3$
35	A	$CO-C_{14}H_{29}$	35−36[a]	2HCl	195−197	$C_{19}H_{41}Cl_2N_3O$
36	B	$CH_2-C_{14}H_{29}$	28−30	3HCl	240−241	$C_{19}H_{44}Cl_3N_3$
37	B	$CO-C_{14}H_{29}$	<30	2HCl	122−123	$C_{19}H_{41}Cl_2N_3O$

[a] Compound has been purified by column chromatography using eluent B (see Experimental Section).

and by the known Gi-protein activators **1**[21] and **5**,[23] used as reference compounds. Considering the multiplicity of GTP-binding proteins present in lymphocytes,[51] it may seem difficult to evidence a particular G-protein subtype activation with the use of a GTP analogue. However, not only a preferential affinity of Gpp(NH)p toward Gi proteins in the range of concentrations used has been evidenced,[52,53] but also a widely different expression of each type of G protein is documented, indicating that Gi are commonly 10 times more abundant than Gs.[54] Therefore, Gpp(NH)p appears to be a suitable tool to reproduce a physiological Gi protein activation. Preincubation of lymphocytes with pertussis toxin at a concentration consistent with a complete ADP-ribosilation of Gi proteins[55] prevented the inhibi-

tion of the forskolin-stimulated adenylyl cyclase activity (data not shown), suggesting that the effects observed were related to a Gi protein system activation.

Results

GTPγS(^{35}S) Binding to Recombinant Gi Proteins. Gi protein activation induces the exchange of GDP for GTP on the α subunit. As a result, α-GTP dissociates from βγ subunits. Therefore, the evaluation of GTPγS binding to the α subunit is a good method to ascertain the ability of tested compounds to activate Gi protein. Stimulation of binding at 5×10^{-5} M (percent of basal/30 min) was taken as a measure of efficacy and EC_{50} (determined using concentrations of 1×10^{-4}, 5×10^{-5}, 3×10^{-5}, 2×10^{-5}, 1×10^{-5}, 5×10^{-6}, 1×10^{-6}, and 1×10^{-7} M of each compound) as a measure of potency (Table 5). To compare the efficacy of the tested molecules we chose the maximum effect obtainable at the concentration of 5×10^{-5} M, because higher concentrations, although more active, showed a higher aspecific binding. To test the method specificity we first evaluated the effect of (i) a compound (morphine) that activates Gi protein in a receptor-mediated manner, (ii) a compound (atenolol) that blocks receptor-mediated Gs activation, and (iii) a compound (AMPA) that modulates Na^+ channels. All three compounds were completely inactive (data not shown). As far as reference compound **1** is concerned, we confirmed its effect on α_{i1} and α_{o1} subunits and demonstrated its activity also on α_{i3}. Instead, reference compound **5**, which had never been tested before by this method, showed little activity on α_{i1} and α_{o1} but was able to stimulate GTPγS binding on α_{i3} (Table 5). Figure 1 shows a comparison of the effects of increasing concentrations of compound **1** and compounds **34** and **36** on $G\alpha_{i1}$ (panel A), $G\alpha_{i3}$ (panel B), and $G\alpha_{o1}$ (panel C) isoforms. Among the newly synthesized compounds, comparing the effect at 5×10^{-5} M, we found that compounds **8**, **18**, **22**, **25**, **28**, **31a**, **31b**, **32**, **34**, **36**, and **37** were superior to compound **1** in increasing GTPγS binding. Interestingly, compounds **7**, **18**, **30**, and **32** were particularly active on $G\alpha_o$, and compound **8** was notably active on $G\alpha_i$. Compounds **7**, **13**, **23**, **24**, **27**, **29**, and **30** showed an activity roughly comparable to that of **1**, whereas compounds **6**, **9**, **12**, **14**, **16**, **17**, **20**, **21**, and **35** were less active than **1**. All active compounds exerted their effect at $EC_{50} > 1 \times 10^{-6}$ M (Table 5). Compounds **10**, **11**, **15**, and **19** were completely inactive.

Protein Gi Activation. Adenylyl cyclase activity was stimulated by using the diterpene forskolin at a concentration of 10^{-4} M. A relative value of 100% (100.1 ± 4.7) was assigned to the concentration of cAMP obtained after forskolin stimulation. The inhibition of the stimulated cAMP production by Gi proteins was then evaluated by administration of Gpp(NH)p, a nonhydrolyzable GTP analogue used as positive control, in the concentration range of 10^{-10}–10^{-4} M. The results, reported in Figure 2, are expressed as percent of stimulated cAMP production relative to control (10^{-4} M forskolin). Gpp(NH)p produced a dose-dependent inhibition of forskolin-stimulated adenylyl cyclase activity detectable by a reduction of cAMP levels of ~35% (Figure 2A). Similarly to Gpp(NH)p, also the reference compounds **1** and **5** produced a dose-dependent reduction of cAMP levels (Figure 2A). Similar results were obtained when the experiments were carried out on permeabilized lymphocytes (data not shown). At the concentration of 10^{-10} M they were both inactive; statistical significance was reached at 10^{-7} M, and their maximum effect was at 10^{-5} M. The effect observed was prevented by pretreatment with pertussis toxin, indicating that the cAMP level reduction is due to a Gi protein system activation (data not shown). Among the molecules investigated, different pharmacological profiles were observed (Figure 2). Compounds **7**, **9**, and **36** inhibited cAMP production, reaching statistical significance at 10^{-7} M (Figure 2B,C). The intensity of the effect produced was similar to that produced by the reference drugs **1** and **5**. Compound **7** appeared to be one of the most active, but its chloromethyl derivative, **23**, which carries a permanent charge on the nitrogen, showed a much lower ability to reduce the cAMP levels (Figure 2C). Compounds **24** and **34** were able to inhibit the cAMP production with an efficacy similar to that of the reference drugs, but the statistical significance was reached at 10^{-6} M (Figure 2D). A reduction of the cAMP production was also observed with compounds **13**, **29**, and **31b**, which showed statistical significance at 10^{-5} M (Figure 2E). Finally, a Gi protein-activating effect was also observed for compounds **16**, **18**, **20**–**22**, **25**, **30**, and **32** even if the reduction of cAMP production was significant only at 10^{-4} M (Figure 2F).

Compounds **6**, **8**, **10**–**12**, **14**, **15**, **17**, **19**, **27**, **28**, **31a**, **33**, **35**, and **37** were unable to reduce the cAMP production at all concentrations tested (data not shown). Among them, compounds **27**, **28**, and **31a** were unable to produce any modification on cAMP levels also in permeabilized cells, ruling out the possibility that their inactivity was due to a poor ability to cross the plasma membranes (data not shown).

Discussion

Compounds **7**, **9**, **13**, **18**, **20**–**22**, **24**, **25**, **29**, **30**, **31b**, **32**, **34**, and **36** were able to stimulate GTPγS binding and to inhibit cAMP production. The efficacy of the above-mentioned compounds in inhibiting the adenylyl cyclase activity was similar to that of the reference drugs because the highest reduction of cAMP production was reached at 10^{-5}–10^{-4} M, in correspondence to the maximum effect produced by Gpp(NH)p. Among them, compounds **7**, **9**, **24**, **34**, and **36** showed the highest potency, being active at 10^{-7}–10^{-6} M. Therefore, in agreement with the good correlation between the results of the two methods, it is very likely that these compounds act through a direct activation of Gi proteins. This is also suggested by the behavior of **23**, the chloromethylated form of **7** (see Scheme 3) that, having a permanent charge on the nitrogen, should cross less efficiently cell membranes, at least up to 10^{-5} M. As a matter of fact, **23** shows a much lower activity on lymphocytes than **7**, which is able to reduce cAMP production at concentrations 1000 times lower. In support of such a hypothesis, **23** showed an activity similar to that of **7** on GTPγS binding where, the test being performed on isolated Gi/o proteins, no membrane crossing is necessary. In this respect, it can be hypothesized that a specific polyamine transporter might be involved in the process. At the moment, we have no

Table 5. Stimulation of [^{35}S]GTPγS Binding to Gi Protein Isoforms by Compounds **6**−**37**

[^{35}S]GTPγS binding on G$\alpha_{i/o}$ subunit	
Gα isoform	basal/30 min
α_{i1}	100 ± 14
α_{i3}	100 ± 37
α_{o1}	100 ± 23

	[^{35}S]GTPγS binding on G$\alpha_{i/o}$ subunit				[^{35}S]GTPγS binding on G$\alpha_{i/o}$ subunit		
no.	Gα isoform	stimulation at 5 × 10^{-5} M % of basal/30 min[a]	EC_{50} (μM)	no.	Gα isoform	stimulation at 5 × 10^{-5} M % of basal/30 min[a]	EC_{50} (μM)
1	α_{i1}	900 ± 85*	47.9 ± 2.0	**22**	α_{i1}	1539 ± 57*	32.4 ± 14.7
	α_{i3}	1030 ± 137*	43.9 ± 1.4		α_{i3}	2275 ± 131*	14.4 ± 0.3
	α_{o1}	2510 ± 118*	28.8 ± 0.7		α_{o1}	3037 ± 172*	13.5 ± 1.7
5	α_{i1}	60 ± 7		**23**	α_{i1}	1576 ± 187*	14.7 ± 0.7
	α_{i3}	391 ± 34*	38.8 ± 6.4		α_{i3}	1626 ± 150*	16.0 ± 0.3
	α_{o1}	129 ± 22			α_{o1}	2086 ± 205*	10.1 ± 0.3
6	α_{i1}	755 ± 45*	21.1 ± 1.7	**24**	α_{i1}	1484 ± 166*	36.4 ± 13.5
	α_{i3}	406 ± 29*	21.2 ± 4.0		α_{i3}	2005 ± 97*	18.8 ± 2.9
	α_{o1}	913 ± 96*	27.1 ± 16.1		α_{o1}	2349 ± 204*	25.5 ± 5.2
7	α_{i1}	1510 ± 166*	19.1 ± 7.6	**25**	α_{i1}	3368 ± 173*	11.8 ± 1.3
	α_{i3}	1640 ± 82*	18.7 ± 6.8		α_{i3}	3443 ± 263*	11.2 ± 0.9
	α_{o1}	1810 ± 133*	6.2 ± 1.1		α_{o1}	3826 ± 218*	13.3 ± 1.4
8	α_{i1}	5694 ± 173*	35.7 ± 7.1	**26**[b]	α_{i1}		
	α_{i3}	5658 ± 225*	41.5 ± 10.9		α_{i3}	nd[e]	nd
	α_{o1}	3476 ± 345*	13.4 ± 0.1		α_{o1}		
9	α_{i1}	704 ± 55*	18.7 ± 2.5	**27**	α_{i1}	1270 ± 76*	50.2 ± 11.0
	α_{i3}	435 ± 26*	18.9 ± 6.6		α_{i3}	1287 ± 64*	34.3 ± 7.1
	α_{o1}	420 ± 11*	19.3 ± 2.4		α_{o1}	1366 ± 216*	22.7 ± 4.4
10	α_{i1}	97 ± 14	-	**28**	α_{i1}	3346 ± 97*	22.1 ± 2.7
	α_{i3}	80 ± 8			α_{i3}	3775 ± 69*	27.6 ± 2.5
	α_{o1}	93 ± 21			α_{o1}	6893 ± 79*	23.0 ± 1.4
11	α_{i1}	81 ± 10		**29**	α_{i1}	1051 ± 69*	25.5 ± 6.7
	α_{i3}	97 ± 14			α_{i3}	1071 ± 56*	25.1 ± 9.3
	α_{o1}	99 ± 22			α_{o1}	1279 ± 53*	25.8 ± 6.9
12	α_{i1}	950 ± 75*	17.6 ± 3.9	**30**[c]	α_{i1}	973 ± 314*	6.4 ± 0.2
	α_{i3}	1178 ± 24*	12.1 ± 2.8		α_{i3}	986 ± 175*	5.9 ± 0.5
	α_{o1}	1498 ± 68*	18.1 ± 0.8		α_{o1}	2271 ± 254*	8.2 ± 1.3
13	α_{i1}	1542 ± 225*	8.6 ± 1.0	**31a**	α_{i1}	2629 ± 159*	18.4 ± 3.7
	α_{i3}	1558 ± 125*	9.3 ± 1.4		α_{i3}	2724 ± 103*	19.0 ± 3.7
	α_{o1}	2388 ± 215*	5.6 ± 0.5		α_{o1}	3347 ± 322*	16.9 ± 2.6
14	α_{i1}	612 ± 104*	25.4 ± 2.0	**31b**	α_{i1}	3291 ± 128*	8.6 ± 1.0
	α_{i3}	424 ± 131*	24.4 ± 1.4		α_{i3}	3244 ± 275*	9.3 ± 1.4
	α_{o1}	920 ± 248*	22.6 ± 0.4		α_{o1}	3597 ± 335*	5.5 ± 0.4
15	α_{i1}	84 ± 7		**32**	α_{i1}	2464 ± 179*	10.6 ± 0.4
	α_{i3}	75 ± 15			α_{i3}	1933 ± 133*	11.4 ± 0.4
	α_{o1}	126 ± 23			α_{o1}	4162 ± 481*	12.4 ± 2.8
16	α_{i1}	392 ± 32*	6.0 ± 0.5	**33**	α_{i1}	825 ± 105*	17.8 ± 2.5
	α_{i3}	265 ± 31*	17.9 ± 2.8		α_{i3}	815 ± 65*	23.0 ± 6.8
	α_{o1}	531 ± 26*	34.9 ± 4.1		α_{o1}	942 ± 143*	24.3 ± 7.3
17	α_{i1}	232 ± 48*	23.0 ± 2.9	**34**[d]	α_{i1}	2328 ± 334*	6.5 ± 0.9
	α_{i3}	181 ± 72	23.3 ± 4.1		α_{i3}	2816 ± 351*	6.5 ± 0.3
	α_{o1}	472 ± 63*	21.4 ± 0.8		α_{o1}	2870 ± 565*	3.7 ± 0.3
18	α_{i1}	2457 ± 210*	451 ± 988	**35**	α_{i1}	173 ± 14*	28.6 ± 0.5
	α_{i3}	2404 ± 262*	8050.3 ± 9260		α_{i3}	211 ± 51*	24.6 ± 3.0
	α_{o1}	5009 ± 415*	10.5 ± 2.7		α_{o1}	194 ± 47*	373.9 ± 292
19	α_{i1}	86 ± 13		**36**[d]	α_{i1}	5060 ± 463*	15.9 ± 3.2
	α_{i3}	102 ± 8	-		α_{i3}	5718 ± 293*[f]	19.0 ± 2.9
	α_{o1}	132 ± 16	-		α_{o1}	5577 ± 479*	6.1 ± 0.5
20	α_{i1}	845 ± 141*	6.6 ± 0.1	**37**	α_{i1}	2322 ± 221*	38.5 ± 3.4
	α_{i3}	1093 ± 131*	6.3 ± 0.3		α_{i3}	3690 ± 280*	24.9 ± 1.1
	α_{o1}	724 ± 293*	6.1 ± 0.1		α_{o1}	4636 ± 292*	23.4 ± 2.2
21	α_{i1}	577 ± 40*	20.1 ± 6.0				
	α_{i3}	815 ± 66*	6.1 ± 0.4				
	α_{o1}	742 ± 43*	12.5 ± 0.1				

[a] These values are taken as a measure of efficacy in stimulating binding. *, $P < 0.01$. [b] Compound does not solubilize. [c] Compound is sparingly soluble at 10^{-5} M. [d] Compound is sparingly soluble at 10^{-4} M. [e] Not done. [f] Experiment is performed at 3 × 10^{-5} M.

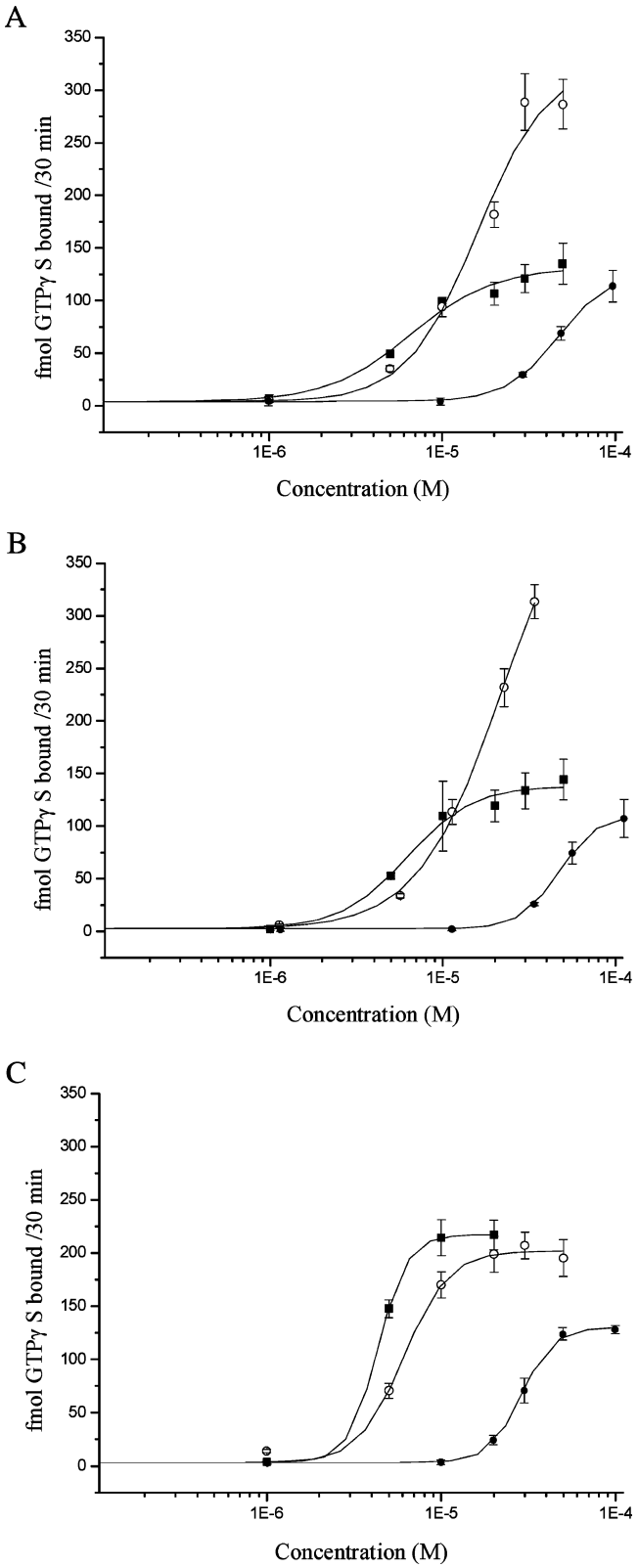


Figure 1. Effect of increasing concentrations of compounds **1** (●), **34** (■), and **36** (○) on the stimulation of [^{35}S]GTPγS binding on Gα_{i1} (A), Gα_{i3} (B), and Gα_{o1} (C). Data are expressed as mean ± SEM.

indication that this is indeed the case, but this possibility cannot be ruled out.

Compounds **6**, **8**, **12**, **14**, **17**, **27**, **28**, **31a**, **33**, and **37** were inactive in inhibiting cAMP production in lymphocytes but effective in stimulating GTPγS binding. This discrepancy might be due to the lack of selectivity of these compounds that, besides Gi proteins, could interact with other targets that would oppose the Gi protein-inhibiting effect. For instance, they might interact with other G protein subtypes, such as Gs proteins, producing a stimulation of adenylyl cyclase which, in its turn, would lead to an increase of the intracellular cAMP contents. It should also be taken into account that their lack of activity on cells might be due to their difficulties in crossing the cell membrane or their poor solubility. On the basis of the results obtained in both assays, the most interesting compounds of the series appear to be **7**, **9**, **22**, **25**, **30**, **31b**, **32**, **34**, and **36**, which seem to be able to cross the cell membrane and show good efficacy and potency in the low micromolar range. Among them, compound **34** appears to be the most potent and **36** the most efficacious. Some subtype selectivity also seems to be present in some of these compounds, in particular **7**, **18**, **30**, and **32**, which show almost a 2-fold efficacy on the α_o subunit with respect to the others.

Establishing structure−activity relationships (SAR) was not the main goal of this research, yet a few correlations between the structure of our compounds and Gi-activating properties can be made. First of all, we were able to confirm some of the molecular requisites that have already been proposed for drugs activating Gi proteins.[21,36] As a matter of fact, with the exceptions of **30** and **31b**, the most active compounds present two or three basic amino groups that are very likely protonated under physiological conditions. In this respect, it is interesting to note that although **31a** (two basic centers) and **31b** (one basic center) are equally active in facilitating GTPγS binding, **31a** does not present any activity on cells, a finding that would suggest that it has problems in crossing the membrane. However, cell permeabilization did not produce any modification on cAMP levels, thus ruling out this possibility. At the moment, we do not have a sound explanation for the different behaviors of **31a** and **31b**. It is also important to note that a secondary terminal nitrogen seems to be preferable for Gi-modulating activity as *N*-methyl derivatives present little or no activity and none of them is present in the list of the most interesting compounds. A second feature, shared by our compounds with drugs known to act directly on Gi proteins, is the presence of a long lipophilic carbon chain. In this respect, even if the length of the methylene chains used in the present research did not vary much, the *n*-pentadecyl chain seems to be the most useful to induce Gi-activating properties.

We found active compounds in all four groups of structures synthesized, which vary essentially in the relative position of the basic nitrogen atoms. This seems to suggest that the arrangement of the basic nitrogen atoms is not crucial for activity, whereas it may contribute to subtype selectivity. However, other research and more dedicated SAR studies will be necessary to confirm these aspects.

Going briefly into details, among the 4,4′-piperidinyl piperidine derivatives, compound **7** is the most active, those with an amido group or with shorter methylene chains being inactive or feebly active. An interesting feature of **7** is its selectivity toward the α_o subtype, in both efficacy and potency. On the contrary, compound

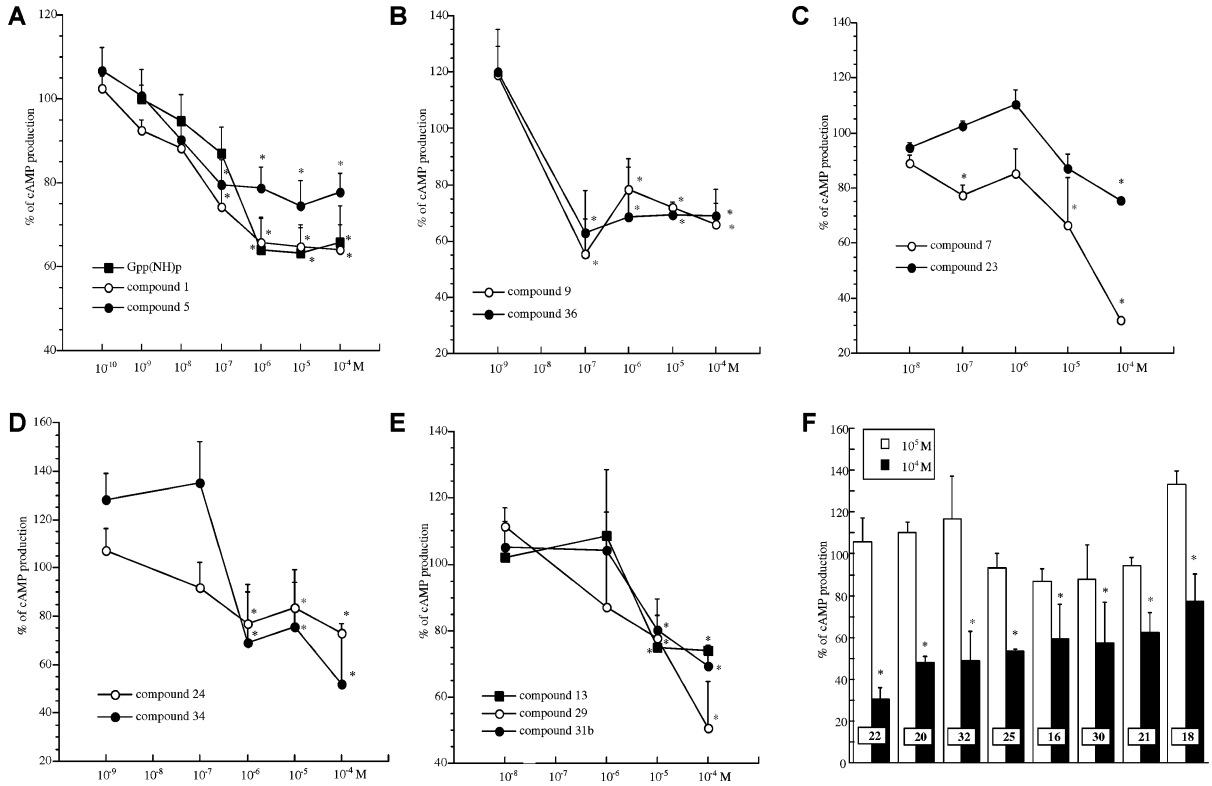


Figure 2. Evaluation of the capability to inhibit the stimulated adenylyl cyclase activity by the investigated compounds. Adenylyl cyclase activity was stimulated by 10^{-4} M forskolin: (A) dose-dependent reduction of cAMP production by the reference drugs [Gpp(NH)p, compounds **1** and **5**]; (B) effect on cAMP production by compounds **9** and **36**; (C) effect on cAMP production by compounds **7** and **23**; (D) effect on cAMP production by compounds **24** and **34**; (E) effect on cAMP production by compounds **13**, **29**, and **31b**; (F) effect on cAMP production by compounds **22**, **20**, **32**, **25**, **16**, **30**, **21**, and **18**. Data are expressed as mean ± SEM relative to control (10^{-4} M forskolin), which was assigned a relative value of 100% (100.1 ± 4.7). Each data point represents the mean of at least three independent experiments. Experiments were performed in duplicate. *, $P < 0.05$ in comparison with forskolin-stimulated lymphocytes.

8, which carries a longer methylene chain, is one of the most efficacious in activating GTPγS binding but shows the opposite selectivity and does not activate Gi proteins in lymphocytes. Introduction of a NH group between the piperidine rings (**22**) maintained activity, whereas introduction of a two-methylene chain (**18**) reduced potency but maintained selectivity for the α_o subtype.

Among the 4-aminopiperidine derivatives (Table 2) compound **25** is the most interesting, in terms of both potency and efficacy; even in this case, reduction (**24**) of the length of the methylene chain reduces activity. The extended chain derivative **26** could not be tested because of solubility problems.

Among the derivatives of piperidine-4-carboxylic acid (Table 3) is found the only active compound possessing only one basic nitrogen (**30**), which combines activity and selectivity toward the α_o subtype. However, confirming that basic nitrogen atoms are important for activity, reduction of the carbonyl group of **30** to give **32** increases activity with little impact on selectivity. Accordingly, introduction of an ethylamino group as in **31a** and **31b** results in two compounds active in stimulating GTPγS binding.

Finally, in the *N*-aminopiperazine group (Table 4) are found two of the most interesting drugs of the series. Compound **34** shows a good efficacy and is the most potent compound studied, particularly on the α_o subtype; its regioisomer, **36**, is the most efficacious. Both, however, do not show selectivity, suggesting that this arrangement of the basic nitrogen atoms is less useful than the other ones to exalt this property.

In conclusion, we have identified a number of compounds with different chemical structures that seem to be able to directly activate Gi proteins and that will be useful for developing new pharmacological tools to study these essential players in the transmission of information between cells and, eventually, to develop clinically useful drugs.

Experimental Section

Chemistry. All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 681 spectrophotometer in Nujol mull for solids and neat for liquids. Unless otherwise stated, NMR spectra were recorded on a Gemini 200 spectrometer (200 MHz for ^{1}H NMR, 50.3 MHz for ^{13}C), and chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063−0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040−0.063 mm; Merck). When necessary, chromatographic separations were performed on an Al_2O_3 column by gravity chromatography (aluminum oxide 90 standardized, Merck). Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values. Eluents used in the purification step were (A) petroleum ether/Et_2O/CH_2Cl_2/absolute EtOH/NH_4OH, 450:180:180:45:2.5; (B) CH_2Cl_2/absolute EtOH/petroleum ether/NH_4OH, 340:65:60:8; (C) petroleum ether/Et_2O/CH_2Cl_2/absolute EtOH/NH_4OH, 900:360:360:180:9.9; and (D) CH_2Cl_2/MeOH/NH_4OH, 50:50:3. Melting points, yields, and column eluents of intermediates are reported in Table 6.

Table 6

no.	mp (°C) base[a]	yield (%)	column eluent
38[b]		45	
39		42	D
40	30−31	62	A
41	33−32	61	A
42	30−31	64	A
43	42−43	41	A
44	35−37	70	A
45	31−33	63	A
46	35−38	86	A
47	33−34	55	A
48[c]	30−31	49	C
49	34−35	64	B
50	140−142[d]	99	
51	oil	99	A and C
52	<30	62	B
53	<30	76	A and C
54	oil	94	
55	oil	92	
56[e]	oil	91	
57	47−50	56	A
58	50−51	71	A and B
59	oil	74	C
60	oil	69	C
61	31−32	21	B
62	56−57	85	C
63[f]	oil	33	B
64	30−32	70	A
65	<30	84	B

[a] Intermediates were identified through IR and NMR spectra; microanalyses were not performed. [b] See ref 23. [c] See ref 39. [d] Melting point of the iodomethylate. [e] See ref 41. [f] See ref 46.

***tert*-Butyl-(1′-tridecyl)-[4,4′]-bipiperidinyl-1-carboxylate (40).** To *tert*-butyl-[4,4′]-bipiperidinyl-1-carboxylate **38**[23] in anhydrous DMF (28 mL × g) were added 2 equiv of NEt_3 and 2 equiv of bromotridecane. After 8 h at reflux, the solvent was removed, the residue made alkaline with $NaHCO_3$, and the solution extracted with CH_2Cl_2. Following anhydrification and evaporation of the solvent, the residue was purified by column chromatography using eluent A to obtain **40** in 62% yield: IR (Nujol) ν 1691 (OCON) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.06−1.21 (m, 6H, CH_2 bipiperidine), 1.21−1.37 (m, 20H, $10CH_2C$), 1.35−1.56 (m, 2H, CH_2CH_2N), 1.46 (s, 9H, CH_3C), 1.60−1.75 (m, 4H, CH_2 bipiperidine + 2CH), 1.75−1.91 (m, 2H, CH_2N bipiperidine), 2.28 (t, J = 7.7 Hz, 2H, CH_2CH_2N), 2.55−2.72 (m, 2H, $2CH_{eq}NBOC$), 2.91−3.04 (m, 2H, CH_2N bipiperidine), 4.04−4.19 (m, 2H, $2CH_{ax}NBOC$). Anal. ($C_{28}H_{54}N_2O_2$) C, H, N.

With the same method described for **40**, starting from **38** or **39** and the proper bromoalkane or alkanoyl chloride, compounds **41**−**47** were obtained.

1-Tridecyl-[4,4′]-bipiperidine (6). Two equivalents of 6 N HCl was added to **40** dissolved in a few milliliters of ethyl acetate and left for 12 h at room temperature. The mixture was basified with 10% NaOH in H_2O and extracted with CH_2Cl_2; anhydrification and evaporation of the solvent gave **6** in quantitative yield: IR (Nujol) ν 3230 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 6.9 Hz, 3H, CH_3CH_2), 1.01−1.21 (m, 6H, CH_2 bipiperidine), 1.21−1.40 (m, 20H, $10CH_2C$), 1.41−1.56 (m, 2H, CH_2CH_2N), 1.61−1.75 (m, 5H, CH_2 bipiperidine + 2CH + NH), 1.75−1.90 (m, 2H, CH_2N bipiperidine), 2.27 (t, J = 7.9 Hz, 2H, CH_2CH_2N), 2.49−2.64 (m, 2H, CH_2NH), 2.90−3.03 (m, 2H, CH_2N bipiperidine), 3.03−3.15 (m, 2H, CH_2NH); ^{13}C NMR ($CDCl_3$) δ 14.53 (q, CH_3), 23.10 (t, CH_2), 27.53 (t, CH_2), 28.20 (t, CH_2), 29.71 (t, CH_2), 30.02 (t, CH_2), 31.04 (t, CH_2), 32.32 (t, CH_2), 41.75 (d, CH), 41.84 (d, CH), 47.45 (t, CH_2), 54.77 (t, CH_2), 59.74 (t, CH_2). Anal. ($C_{23}H_{46}N_2$) C, H, N.

Compounds **7**, **8**, **12**−**14**, **18**, and **20** were obtained in quantitative yields using the same method as described for **6**.

Compounds **6**−**8**, **12**−**14**, **18**, and **20** were transformed in the hydrochlorides by treatment with EtOH/HCl in Et_2O anhydrous. The salts were recrystallized from absolute EtOH and anhydrous Et_2O.

1-Methyl-1′-tridecyl-[4,4′]-bipiperidine (9). Formic acid (17 equiv) and 40% formaldehyde in H_2O (4.8 equiv) were added to compound **6** in EtOH and refluxed for 4 h. The solution was made alkaline with 10% NaOH and extracted with CH_2Cl_2. After anhydrification, evaporation of the solvent gave **9** as a pure compound: ^1H NMR ($CDCl_3$) δ 0.89 (t, J = 6.9 Hz, 3H, CH_3CH_2), 0.96−1.15 (m, 2H, CH_2 bipiperidine), 1.15−1.41 (m, 24H, $10CH_2C$ + $2CH_2$ bipiperidine), 1.41−1.56 (m, 2H, CH_2CH_2N), 1.61−1.76 (m, 4H, CH_2 bipiperidine + 2CH), 1.76−1.92 (m, 4H, $2CH_2N$ bipiperidine), 2.21−2.32 (m, 2H, CH_2CH_2N), 2.25 (s, 3H, CH_3N), 2.80−2.90 (m, 2H, CH_2N bipiperidine), 2.90−3.01 (m, 2H, CH_2NCH_3); ^{13}C NMR ($CDCl_3$) δ 14.54 (q, CH_3), 23.10 (t, CH_2), 27.54 (t, CH_2), 28.20 (t, CH_2), 29.77 (t, CH_2), 29.91 (t, CH_2), 29.97 (t, CH_2), 30.04 (t, CH_2), 32.33 (t, CH_2), 40.66 (d, CH), 41.33 (d, CH), 46.88 (q, CH_3), 54.75 (t, CH_2), 56.68 (t, CH_2), 59.74 (t, CH_2). Anal. ($C_{24}H_{47}N_2$) C, H, N.

Using the same method as described above and starting from **7**, **8**, **12**−**14**, **18**, and **20**, derivatives **10**, **11**, and **15**−**17** were obtained, respectively. Compounds **10** and **11** were purified by column chromatography using eluents B and C, respectively, whereas compounds **15**−**17**, **19**, and **21** were obtained pure.

Compounds **9**−**11**, **15**−**17**, **19**, and **21** were transformed into the hydrochlorides with the method described above.

(1-Benzyl-4′-piperinyl)-(1-pentadecyl-4′-piperidinyl)-amine (49). One equivalent of **48**, 1 equiv of 4-amino-1-benzylpiperidine, and 1.1 equiv of $(^iPrO)_4Ti$ were left at room temperature until the IR carbonyl absorption disappeared, and then 5 mL of absolute EtOH and 2 equiv of $NaBH_3CN$ were added. After 24 h at room temperature, the solution was made alkaline with 10% NaOH and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave a mixture that was purified by column chromatography using eluent B to give **49** (64% yield): IR (Nujol) ν 3200−3400 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 6.9 Hz, 3H, CH_3CH_2), 1.08−1.30 (m, 24H, $12CH_2C$), 1.30−1.52 (m, 6H, CH_2CH_2N + $2CH_2$ piperidine), 1.72−1.90 (m, 4H, $2CH_2$ piperidine), 1.90−2.08 (m, 4H, CH_2N piperidine), 2.29 (t, J = 7.7 Hz, 2H, CH_2CH_2N), 2.49−2.68 (m, 2H, 2CH), 2.78−2.95 (m, 4H, $2CH_2N$ piperidine), 3.50 (s, 2H, CH_2Ph), 7.20−7.37 (m, 5H, CH aromatics). Anal. ($C_{32}H_{57}N_3$) C, H, N.

Using the same method and starting from **59** and benzylamine, compound **60** was obtained. The compound was obtained by purification of the reaction mixture by column chromatography using eluent C.

[1-Pentadecyl-(4′-piperidyl)]-4-piperidylamine (22). Ten percent Pd/C (0.7 g × 1 g of benzylderivative) was added to **49** in absolute EtOH and hydrogenated at 53 psi for 24 h at room temperature. The carbon was filtered off and the solvent removed under vacuum to obtain pure **22**: IR (Nujol) ν 3200−3380 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 6.9 Hz, 3H, CH_3CH_2), 1.10−1.31 (m, 24H, $12CH_2C$), 1.31−1.56 (m, 6H, CH_2CH_2N + $2CH_2$ piperidine), 1.73−1.93 (m, 4H, $2CH_2$ piperidine), 1.93−2.14 (m, 3H, CH_2N piperidine + NH), 2.29 (t, J = 8.0 Hz, 2H, CH_2CH_2N), 2.50−2.80 (m, 4H, CH_2NH + 2CH), 2.81−2.99 (m, 2H, CH_2N piperidine), 3.04−3.19 (m, 2H, CH_2NH); ^{13}C NMR ($CDCl_3$) δ 14.58 (q, CH_3), 23.14 (t, CH_2), 27.55 (t, CH_2), 28.13 (t, CH_2), 29.78 (t, CH_2), 30.11 (t, CH_2), 32.35 (t, CH_2), 33.35 (t, CH_2), 34.43 (t, CH_2), 45.50 (t, CH_2), 51.45 (d, CH), 53.12 (t, CH_2), 59.28 (t, CH_2). Anal. ($C_{25}H_{51}N_3$) C, H, N.

Using the same method, starting from **51**−**54**, **57**, **58**, and **60**, compounds **25**, **27**, **30**, and **32** were obtained pure, whereas **24**, **26,** and **29** were purified by column chromatography using the eluent reported in Table 2.

***tert*-Butyl-(1′-methyl-1′-pentadecyl-[4,4′]-bipiperidinium iodide)-1-carboxylate (50).** Compound **41** (60 mg) in anhydrous ether was added with an excess of CH_3I and left at room temperature for 48 h. The salt was filtered under vacuum and purified by column chromatography using eluent B: ^1H NMR ($CDCl_3$) δ 0.86 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.10−1.35 (m, 24H, $12CH_2C$), 1.36−1.40 (m, 2H, CH_2CH_2N), 1.43 (s, 9H, CH_3C), 1.62−1.73 (m, 12H, CH_2 piperidine), 1.84−2.02

(m, 2H, CH_2 piperidine), 2.64 (t, J = 7.8 Hz, 2H, CH_2CH_2N), 3.20 (s, 3H, CH_3N), 3.53−3.61 (m, 2H, CH_2 piperidine), 4.08−4.14 (m, 2H, CH_2 piperidine). Anal. ($C_{31}H_{61}IN_2O_2$) C, H, N.

1-Methyl-1-pentadecyl-[4,4′]-bipiperidinium Chloride (23). Two equivalents of 6 M HCl were added to **50** dissolved in CH_3CN. After 2 h at room temperature and 5 min at 60 °C, the solution was made alkaline with 10% NaOH and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave **23** in 30% yield: mp 180−181 °C from anhydrous ether; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.19−1.31 (m, 24H, $12CH_2C$), 1.32−1.39 (m, 2H, CH_2CH_2N), 1.65−1.89 (m, 12H, CH_2 piperidine), 1.91−2.02 (m, 2H, CH_2 piperidine), 2.61 (t, J = 7.7 Hz, 2H, CH_2CH_2N), 3.02−3.14 (m, 2H, CH_2 piperidine), 3.21 (s, 3H, CH_3N), 3.32−3.43 (m, 2H, CH_2 piperidine), 3.65 (bs, 1H, NH). Anal. ($C_{26}H_{53}ClN_2$) C, H, N.

(1-Benzyl-4-piperidinyl)tridecylamine (51). Two equivalents of NEt_3 and 1 equiv of bromotridecane were added to 4-amino-1-benzylpiperidine in CH_3CN. After 3 h at reflux, the solvent was removed, the residue made alkaline with 10% NaOH, and the solution extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave a mixture from which **51** was purified by flash chromatography using the eluent reported in Table 6: IR (neat) ν 3300 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.21−1.37 (m, 20H, $10CH_2C$), 1.37−1.57 (m, 4H, CH_2CH_2NH + CH_2 piperidine), 1.80−1.93 (m, 2H, CH_2 piperidine), 1.93−2.09 (m, 2H, CH_2N), 2.39−2.53 (m, 1H, CH), 2.62 (t, J = 7.3 Hz, 2H, CH_2CH_2NH), 2.80−2.93 (m, 2H, CH_2N), 3.50 (s, 2H, CH_2Ph), 7.21−7.38 (m, 5H, CH aromatics). Anal. ($C_{25}H_{44}N_2$) C, H, N.

Using the method described above, starting from ethyl isonipecotate, 1-piperidone hydrochloride monohydrate, 1-nitrosopiperazine, 1-amino-4-benzylpiperazine, **30**, and **63**[46] and using the proper bromoalkane or alkanoyl chloride, compounds **48**,[39] **52**, **53**, **59**, **61**, and **62** were obtained, which were purified by flash chromatography using the eluent reported in Table 6. Compounds **65** and **64** were purified by column chromatography (eluent in Table 6), whereas compounds **31a**, **54**, and **55** were obtained pure.

In the same reaction of **30**, when DMF was used instead of CH_3CN, compound **31b** was obtained: IR (Nujol) ν 3300−3200 (NH), 1645 (NHCOH), 1633 (CONH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.8 Hz, 3H, CH_3CH_2), 1.20−1.37 (m, 24 H, 12 CH_2C), 1.41−1.56 (m, 2H, CH_2CH_2N), 1.66−1.92 (m, 4H, $2CH_2$-piperidine), 1.94−2.08 (m, 3H, CH_2N + CH), 2.42 (t, J = 6.3 Hz, 2H, NCH_2CH_2NH), 2.78−2.98 (m, 2H, CH_2N), 3.18−3.26 (m, 2H, CH_2NHCO), 3.28−3.41 (m, 2H, CH_2NHCHO), 5.60 (bs, 1H, NH), 6.18 (bs, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 14.48 (q, CH_3), 23.12 (t, CH_2), 27.34 (t, CH_2), 29.38 (t, CH_2), 29.73 (t, CH_2), 29.78 (t, CH_2), 30.11 (t, CH_2), 32.33 (t, CH_2), 35.04 (t, CH_2), 39.72 (t, CH_2), 39.85 (t, CH_2), 43.75 (d, CH), 53.31 (t, CH_2), 53.54 (t, CH_2), 56.80 (t, CH_2), 161.56 (d, CHO), 175.04 (s, CO). Anal. ($C_{24}H_{47}N_3O_2$) C, H, N.

1-Benzylpiperidine-4-carboxylic acid pentadecylamide (57). To *N*-benzylisonipecotic acid **56**[41] (0.55 g; 2.1 mmol) in $CHCl_3$, cooled to 0 °C and under nitrogen was added 0.39 mL (5.4 mmol) of NEt_3 and 3.1 mL (3.2 mmol) of ethyl chloroformate. After 1 h, pentadecylamine (0.73 g; 3.2 mmol) in a few milliliters of $CHCl_3$ was added, and the mixture was kept at room temperature for 20 min. The solution was then basified with $NaHCO_3$ and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave a residue that was purified by chromatography using eluent A, to give **57** (yield 56%): mp 47−50 °C; IR (Nujol) ν 3307 (NHCO), 1640 (CONH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.17−1.40 (m, 24H, $12CH_2C$), 1.40−1.59 (m, 2H, CH_2CH_2NH), 1.67−1.91 (m, 4H, $2CH_2$ piperidine), 1.91−2.18 (m, 3H, CH_2N + CH), 2.86−3.00 (m, 2H, CH_2N), 3.23 (m, 2H, CH_2CH_2NH), 3.49 (s, 2H, CH_2Ph), 5.57 (bt, 1H, NH), 7.20−7.39 (m, 5H, CH aromatics). Anal. ($C_{28}H_{48}N_2O$) C, H, N.

[1-(2-Aminoethyl)-4-piperidylmethyl]pentadecylamine (33). Borane methyl sulfide complex (2 M in THF; 2.2 mmol) was added to **31a** (0.75 mmol) in THF anhydrous at reflux under nitrogen. After 1 h, the solution was treated with 4.9 mmol of 6 M HCl and maintained at reflux for 30 min. The cold solution was made alkaline with 10% NaOH and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave a mixture that was purified by column chromatography with the eluent reported in Table 3 to give **33**: ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.18−1.32 (m, 24H, CH_2−C), 1.35 (bs, 2H, NH_2), 1.43−1.55 (m, 2H, CH_2−CH_2−NH_2), 1.63−1.74 (m, 3H, CH_2 piperidine + CH), 1.86−2.02 (m, 2H, CH_2 piperidine), 2.38 (t, J = 6.1 Hz, 2H, $CHCH_2NH_2$), 2.47 (d, J = 4.0 Hz, 2H, NCH_2CH), 2.50−2.61 (m, 2H, CH_2 piperidine), 2.78 (t, J = 6.1 Hz, 2H, $CH_2CH_2NH_2$), 2.83−2.95 (m, 4H, CH_2 piperidine + NH_2CH_2C); ^{13}C NMR ($CDCl_3$) δ 17.69 (q), 23.12 (t), 27.78 (t), 29.78 (t), 30.04 (t), 30.11 (t), 31.04 (t), 32.35 (t), 36.35 (d), 39.38 (t), 50.54 (t), 54.22 (t), 56.18 (t), 61.63 (t). Anal. ($C_{23}H_{49}N_3$) C, H, N.

Using the same method and starting from **57**, compound **58** was obtained and purified by flash chromatography.

1-Pentadecyl-4-piperidinylamine (28). To 1 equiv of **48** in anhydrous MeOH were added molecular sieves (3 Å) (1 g of derivative × 8 g of molecular sieves), 10 equiv of ammonium acetate, and 1 equiv of $NaBH_3CN$, under N_2. After 24 h at room temperature, the solvent was removed, 5 mL of H_2O was added, and the solution was adjusted at pH 2 with 6 N HCl. After evolution of gas had ceased, the solution was neutralized with KOH, the salts were filtered off, and the solution was extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave **28** pure: IR (Nujol) ν 3300−3400 (NH_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, J = 6.9 Hz, 3H, CH_3CH_2), 1.15−1.39 (m, 26H, $12CH_2C$ + CH_2 piperidine), 1.39−1.55 (m, 2H, CH_2CH_2N), 1.73−1.89 (m, 2H, CH_2 piperidine), 1.89−2.07 (m, 2H, CH_2N piperidine), 2.08 (bs, 2H, NH_2), 2.29 (t, J = 8.0 Hz, 2H, CH_2CH_2N), 2.56−2.73 (m, 1H, CH), 2.79−2.94 (m, 2H, CH_2N piperidine); ^{13}C NMR ($CDCl_3$) δ 14.56 (q, CH_3), 23.12 (t, CH_2), 27.58 (t, CH_2), 28.15 (t, CH_2), 29.78 (t, CH_2), 30.02 (t, CH_2), 30.09 (t, CH_2), 32.33 (t, CH_2), 36.32 (t, CH_2), 49.19 (d, CH), 53.00 (t, CH_2), 59.28 (t, CH_2). Anal. ($C_{20}H_{42}N_2$) C, H, N.

Using the same method, starting from **59**, compound **29** was obtained.

4-Pentadecylpiperazin-1-ylamine (34). Compound **61** (0.58 mmol) in 0.6 mL of H_2O and 0.3 mL of MeOH cooled at 0 °C was added to 0.7 mmol of concentrated HCl and 1.5 mmol of Zn. After a while, 3.2 mmol of concentrated HCl was added, keeping the mixture for 30 min at room temperature. Finally, the suspension was refluxed for 2 h. The mixture was then basified with 50% NaOH and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent gave a mixture that was purified by column chromatography with the eluent reported in Table 4 to give **34**: IR (Nujol) ν 3300−3600 (NH_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 6.9 Hz, 3H, CH_3CH_2), 1.16−1.38 (m, 24H, $12CH_2C$), 1.40−1.64 (m, 2H, CH_2CH_2N), 1.58 (bs, 2H, NH_2), 2.31 (t, J = 8.0 Hz, 2H, CH_2CH_2N), 2.36−2.48 (m, 4H, $2CH_2$ piperazine), 2.91 (t, J = 5.1 Hz, 4H, $2CH_2$ piperazine); ^{13}C NMR ($CDCl_3$) δ 14.56 (q, CH_3), 23.12 (t, CH_2), 27.11 (t, CH_2), 28.05 (t, CH_2), 29.78 (t, CH_2), 30.02 (t, CH_2), 30.09 (t, CH_2), 32.33 (t, CH_2), 46.45 (t, CH_2), 55.02 (t, CH_2), 59.90 (t, CH_2). Anal. ($C_{19}H_{41}N_3$) C, H, N.

With the same method, starting from **62**, compound **35** was obtained. Both compounds were transformed into the hydrochlorides as described before.

Pentadecylpiperazin-1-ylamine (36). Five equivalents of $HCOONH_4$ and 0.5 equiv of 10% Pd/C were added to a solution of **64** in anhydrous MeOH under N_2. The mixture was refluxed for 8 h, then the carbon was filtered off and the solution was evaporated. The residue was made alkaline with 10% NaOH and extracted with CH_2Cl_2. Anhydrification and evaporation of the solvent afforded **36** pure: IR (Nujol) ν 3200−3400 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H, CH_3CH_2), 1.13−1.38 (m, 24H, $12CH_2C$), 1.40−1.63 (m, 3H, CH_2CH_2N + NH), 2.30 (t, J = 7.9 Hz, 2H, CH_2CH_2N), 2.36−2.49 (m, 4H, $2CH_2NH$ piperazine), 2.89 (t, J = 5.1 Hz, 4H, $2CH_2NNH$); ^{13}C NMR ($CDCl_3$) δ 14.56 (q, CH_3), 23.12 (t, CH_2), 27.09 (t, CH_2), 28.07 (t, CH_2), 29.78 (t, CH_2), 30.02 (t, CH_2), 30.09 (t, CH_2), 32.33 (t, CH_2), 46.48 (t, CH_2), 55.04 (t, CH_2), 59.92 (t, CH_2). Anal. ($C_{19}H_{41}N_3$) C, H, N.

With the same method, starting from **65**, pure **37** was obtained.

Experimental Pharmacology

Isolation of Human Lymphocytes. Peripheral blood samples (15−20 mL) were immediately treated with heparin sodium (500 IU of heparin/10 mL of blood) to prevent coagulation and diluted 1:1 with saline solution. The lymphocytes were then isolated by subjecting the diluted blood to Ficoll-isopaque (istopaque) density-gradient centrifugation as described by Böyum.[56] Mononuclear cells were counted.

Adenylyl Cyclase Activity Assay in Intact Lymphocytes. The activation of Gi proteins was assessed by evaluating the ability of the investigated compounds to inhibit stimulated adenylyl cyclase activity in peripheral blood intact lymphocytes. Lymphocytes from healthy subjects were preincubated with or without pertussis toxin (PTX) at the concentration of 100 ng/mL at 37 °C for 90 min as previously described.[50] For the determination of adenosine 3′,5′-cyclic monophosphate (cAMP) content, lymphocytes [$(1-2) \times 10^6$ intact cells/assay] were incubated either with dimethyl sulfoxide (DMSO) or 10^{-4} M forskolin, which stimulated the cAMP formation.[50] To obtain the inhibition curve of cAMP production, the nonhydrolyzable analogue of GTP, Gpp(NH)p (guanylyl 5′-imidophosphate), or the investigated molecules were added to the forskolin-containing samples in concentrations ranging from 1 nM to 100 μM, in a final volume of 300 μL. After a 15-min incubation at 37 °C, the cells were lysed and the samples centrifuged according to the method of Brodde et al.[57] The cAMP content was determined in a 100-μL aliquot of the supernatant using an enzyme immunoassay kit (Amersham). Protein concentration was determined by the bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin as standard. All assays were performed in duplicate. The recovery of a known amount of cAMP (10 pmol), added to the incubation mixture after boiling, was found to be 94.2 ± 7.8% ($n = 6$). For compounds **1**, **5**, **27**, **28**, and **31a** the inhibition of the adenylyl cyclase activity was also detected on lymphocytes that were first permeabilized with the detergent digitonin (10 μg/mL) under such conditions to maintain the biological functions intact.[58]

Expression and Purification of G Proteins. The CDS (coding sequence) encoding for α *inhibitory* subunit isoforms 1 ($\alpha_{i/1}$) and 3 ($\alpha_{i/3}$) and for α *other* subunit isoform 1 ($\alpha_{o/1}$) were obtained by RT-PCR technique. Total RNA from human brain and heart (brain for isoforms $\alpha_{i/1}$ and $\alpha_{o/1}$, heart for isoform $\alpha_{i/3}$) was extracted by TriReagent (Sigma) according to the manufacturer's protocol. Five hundred nanograms of total RNA was reverse transcribed and amplified by the SuperScript One-Step RT-PCR system (Invitrogen) (primer sequences were as follows):

α_{i1} UP, 5′→3′ ATGGGCTGCACGCTGAGCG;

DN 5′→3′ CAAGACTCTAGTCTACAGAGATCC;

α_{i3} UP, 5′→3′ ATGGGCTGCACGTTGAGCG;

DN 5′→3′ GTCTGGTCTCAACACTCCACAC;

α_{o1} UP, 5′→3′ ATGGGATGTACTCTGAGCGCAGA;

DN 5′→3′ CAGGACAAGAGGTCAGTACAA-GC

PCRs were performed for 35 cycles using 15 s of denaturation at 94 °C, 30 s of annealing at 60 °C, and 1 min/kilobase extension at 68 °C.

All PCR products were cloned into pCR-TOPO T7 expression vector and transformed into competent *Escherichia coli* BL21/DE3. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 34 μg/mL chloramphenicol. Bacteria were collected by centrifugation at 4 °C for 15 min at 5000 rpm, and cell pellets were resuspended in binding buffer (50 mM Hepes, pH 8.0). The solution was then sonicated on ice with six 10-s bursts at high intensity with a 10-s cooling period between each burst. After centrifugation at 3000*g* for 15 min to precipitate the cellular debris, the supernatant was transferred to a fresh tube and 5 μL of the lysate was removed for SDS-PAGE analysis. Purification was performed by immobilized metal affinity chromatography (IMAC) exploiting the 6xHistidine-tag fused to the recombinant protein.

GTPγS Binding. Binding of GTPγS (the nonhydrolyzable GTP analogue) to recombinant G protein was measured according to a standard method.[22] Three picomoles of α subunit in native conditions or reconstituted into lipoid vesicles was incubated at 30 °C (20 °C for $G\alpha_o$) for 30 min in 100 μL of a reaction mixture containing 50 mM Hepes−NaOH, 1 mM EDTA, 1 mM DTT, 100 μM $MgSO_4$, and 0.1 μM [^{35}S]GTPγS (0.1 μCi/tube). Reaction was stopped by the addition of 1 mL of an ice-cold stop buffer (consisting of 10 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, and 100 mM NaCl). The diluted samples were filtered through cellulose nitrate membranes (0.45-μm pore size) under weak vacuum. The filters were washed with 12 mL of the same buffer and dried. The retained radioactivity was quantified by scintillation spectroscopy. Specific binding was calculated by subtracting the GTPγS bound in the presence of 100 μM cold GTPγS from total binding.

Supporting Information Available: IR and ^{1}H NMR data (chemical shifts, ppm) and shift assignment of compounds **7**−**65**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Freissmuth, M.; Waldhoer, M.; Bofill-Cardona, E.; Nanoff, C. G protein antagonists. *Trends Pharmacol. Sci.* **1999**, *20*, 237−245.
(2) Berman, D. M.; Gilman, A. G. Mammalian RGS proteins: barbarians at the gate. *J. Biol. Chem.* **1998**, *273*, 1269−1272.
(3) Wieland, T.; Chen, C. K. Regulators of G-protein signalling: a novel protein family involved in timely deactivation and desensitization of signalling via heterotrimeric G proteins. *Naunyn Schmiedeberg's Arch. Pharmacol.* **1999**, *360*, 14−26.
(4) Cismowski, M. J.; Ma, C.; Ribas, C.; Xie, X.; Spruyt, M.; Lizano, J. S.; Lanier, S. M.; Duzic, E. Activation of heterotrimeric G-protein signaling by a Ras-related protein. *J. Biol. Chem.* **2000**, *275*, 23421−23424.
(5) Luo, Y.; Denker, B. M. Interaction of heterotrimeric G protein Gαo with Purkinje cell protein-2. Evidence for a novel nucleotide exchange factor. *J. Biol. Chem.* **1999**, *274*, 10685−10688.
(6) Cismowski, M. J.; Takesono, A.; Chienling, M.; Lizano, J. S.; Xie, X.; Fuernkranz, H.; Lanier, S. M.; Duzic, E. Genetic screens in yeast to identify mammalian non receptor modulators of G-protein signaling. *Nat. Biotechnol.* **1999**, *17*, 878−883.
(7) Takesono, A.; Cismowski, M. J.; Ribas, C.; Bernard, M.; Chung, P.; Hazard, S.; Duzic, E.; Lanier, S. M. Receptor-independent activators of heterotrimeric G-protein signaling pathways. *J. Biol. Chem.* **1999**, *274*, 33202−33205.
(8) Lanier, S. M. AGS proteins, GPR motifs and the signals processed by heterotrimeric G proteins. *Biol. Cell* **2004**, *96*, 369−372.
(9) Offermanns, S.; Schultz, G. Complex information processing by the transmembrane signaling system involving G proteins. *N. S. Arch. Pharmacol.* **1994**, *350*, 329−338.
(10) Hohenegger, M.; Waldhoer, M.; Beindl, W.; Boing, B.; Kreimeyer, A.; Nickel, P.; Nanoff, C.; Freissmuth, M. Gsα-selective G protein antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 346−351.
(11) Mousli, M.; Bronner, C.; Bockaert, J.; Rouot, B.; Landry, Y. Interaction of substance P, compound 48/80 and mastoparan with the α-subunit C-terminus of G protein. *Immunol. Lett.* **1990**, *25*, 355−357.
(12) Bueb, J. L.; Mousli, M.; Bronner, C.; Rouot, B.; Landry, Y. Activation of Gi-like proteins, a receptor-independent effect of kinins in mast cells. *Mol. Pharmacol.* **1990**, *38*, 816−822.
(13) Nürnberg, B.; Spicher, K.; Harhammer, R.; Bosserhoff, A.; Frank, R.; Hilz, H.; Schultz, G. Purification of a novel G-protein αo-subtype from mammalian brain. *Biochem. J.* **1994**, *300*, 387−394.
(14) Beindl, W.; Mitterauer, T.; Hohenegger, M.; Ijzerman, A. P.; Nanoff, C.; Freissmuth, M. Inhibition of receptor/G protein coupling by suramin analogues. *Mol. Pharmacol.* **1996**, *50*, 415−423.
(15) Freissmuth, M.; Boehm, S.; Beindl, W.; Nickel, P.; Ijzerman, A. P.; Hohenegger, M.; Nanoff, C. Suramin analogues as subtype-selective G protein inhibitors. *Mol. Pharmacol.* **1996**, *49*, 602−611.

(16) Taylor, J. M.; Neubig, R. R. Peptides as probes for G protein signal transduction. *Cell Signal.* **1994**, *6*, 841−849.
(17) Higashijima, T.; Uzu, S.; Nakajima, T.; Ross, E. M. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). *J. Biol. Chem.* **1988**, *263*, 6491−6494.
(18) Higashijima, T.; Burnier, J.; Ross, E. J. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. *J. Biol. Chem.* **1990**, *265*, 14176−14186.
(19) Hagelüken, A.; Grünbaum, L.; Nürnberg, B.; Harhammer, R.; Schunack, W.; Seifert, R. Lipophilic beta-adrenoceptor antagonists and local anesthetics are effective direct activators of G-proteins. *Biochem. Pharmacol.* **1994**, *47*, 1789−1795.
(20) Naim, M.; Seifert, R.; Nürnberg, B.; Grünbaum, L.; Schultz, A. Some taste substances are direct activators of G-proteins. *Biochem. J.* **1994**, *297*, 451−454.
(21) Leschke, C.; Storm, R.; Breitweg-Lehmann, E.; Exner, T.; Nürnberg, B.; Schunack, W. Alkyl-substituted amino acid amides and analogous di- and triamines: new non-peptide G protein activators. *J. Med. Chem.* **1997**, *40*, 3130−3139.
(22) Breitweg-Lehmann, E.; Czupalla, C.; Storm, R.; Kudlacek, O.; Schunack, W.; Freissmuth, M.; Nürnberg, B. Activation and inhibition of G proteins by lipoamines. *Mol. Pharmacol.* **2002**, *61*, 628−636.
(23) Melchiorre, C.; Bolognesi, M. L.; Budriesi, R.; Ghelardini, C.; Chiarini, A.; Minarini, A.; Rosini, M.; Tumiatti, V.; Wade, E. J. Design, synthesis and biological activity of methoctramine-related polyamines as putative Gi protein activators. *J. Med. Chem.* **2001**, *44*, 4035−4038.
(24) Holz, G. G.; Rane, S. G.; Dunlap, K. GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* **1986**, *319*, 670−672.
(25) Galeotti, N.; Ghelardini, C.; Bartolini, A. Effect of pertussis toxin on morphine, diphenhydramine, baclofen, clomipramine and physostigmine antinociception. *Eur. J. Pharmacol.* **1996**, *308*, 125−133.
(26) Womer, D. E.; DeLapp, N. W.; Shannon, H. E. Intrathecal pertussis toxin produces hyperalgesia and allodynia in mice. *Pain* **1997**, *70*, 223−228.
(27) Jiang, M.; Gold, M. S.; Boulay, G.; Spicher, K.; Peyton, M.; Brabet, P.; Srinivasan, Y.; Rudolph, U.; Ellison, G.; Birnbaumer, L. Multiple neurological abnormalities in mice deficient in the G protein G_o. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3269−3274.
(28) Parenti, M.; Tirone, F.; Giagnoni, G.; Pecora, N.; Parolaro, D. Pertussis toxin inhibits the antinociceptive action of morphine in the rat. *Eur. J. Pharmacol.* **1986**, *124*, 357−359.
(29) Sanchez-Blazquez, P.; Garzon, J. Cholera toxin and pertussis toxin on opioid- and α2-mediated supraspinal analgesia in mice. *Life Sci.* **1991**, *48*, 1721−1727.
(30) Ghelardini, C.; Galeotti, N.; Bartolini, A. Amitriptyline and clomipramine activate Gi protein-signaling pathway in the induction of analgesia. *N. S. Arch. Pharmacol.* **2002**, *365*, 1−7.
(31) Galeotti, N.; Ghelardini, C.; Bartolini, A. Antihistamine analgesia is mediated by Gi-protein activation. *Neuroscience* **2002**, *109*, 811−818.
(32) Sanchez-Blazquez, P.; Juarros, J. L.; Martinez-Peña, Y.; Castro, M. A.; Garzon, J. $G_{x/z}$ and G_{i2} transducer proteins on μ/δ opioid-mediated supraspinal antinociception. *Life Sci.* **1993**, *53*, PL381−PL386.
(33) Raffa, R. B.; Martinez, R. P.; Connelly, C. D. G-protein antisense oligodeoxyribonucleotides and μ-opioid supraspinal antinociception. *Eur. J. Pharmacol.* **1994**, *258*, 5−7.
(34) Raffa, R. B.; Connelly, C. D.; Chambers, J. R.; Stone, D. J. Alpha-subunits G-protein antisense oligodeoxynucleotide effects on supraspinal (icv) α2-adrenoceptor antinociception in mice. *Life Sci.* **1996**, *58*, 77−80.
(35) Galeotti, N.; Ghelardini, C.; Zoppi, M.; Del Bene, E.; Raimondi, L.; Beneforti, L.; Bartolini, A. Hypofunctionality of Gi proteins as aetiopathogenic mechanism for migraine and cluster headache. *Cephalalgia* **2001**, *21*, 38−45.
(36) Mousli, M.; Bueb, J. L.; Bronner, C.; Rouot, B.; Landry, Y. G protein activation: a receptor independent mode of action for cationic amphiphilic neuropeptides and venom peptides. *Trends Pharmacol. Sci.* **1990**, *11*, 358−362.
(37) Fierz-David, H. E.; Kuster, W. Zur kenntnis homologer reihen acylierter azofarbstoffe aus o- und p-acylamino-phenolen und 1,7-acylamino-naphthalen. *Helv. Chim. Acta* **1939**, *22*, 82−112.
(38) Cope, A. C.; LeBel, N. A.; Lee, H.-H.; Moore, W. R. Amine oxides. III. Selective formation of olefins from unsymmetrical amine oxides and quaternary ammonium hydroxides. *J. Am. Chem. Soc.* **1957**, *79*, 4720−4729.
(39) Denmark, S. E.; Forbes, D. C.; Hays, D. S.; DePue, J. S.; Wilde, R. G. Catalytic epoxidation of alkenes with ozone. *J. Org. Chem.* **1995**, *60*, 1391−1407.
(40) Mattson, R. J.; Pham, H. M.; Lenck, D. J.; Cowen, K. A. An improved method for reductive alkylation of amines using titanium(IV) isopropoxide and sodium cyanoborohydride. *J. Org. Chem.* **1990**, *55*, 2552−2554.
(41) Strassler, C.; Linden, A.; Heimgartner, H. Novel heterospirocyclic 3-amino-2*H*-azirines as synthons for heterocyclic α-amino acids. *Helv. Chim. Acta* **1997**, *80*, 1528−1554.
(42) Gilligan, P. J.; Cain, G. A.; Christos, T. E.; Cook, L.; Drummond, S.; Johnson, A. L.; Kergaye, A. A.; McElroy, J. F.; Rohrbach, K. W.; Schmidt, W. K.; Tam, S. W. Novel piperidine σ-receptor ligands as potential antipsychotic drugs. *J. Med. Chem.* **1992**, *35*, 4344−4361.
(43) Johnstone, R. A. W.; Rose, M. E. A rapid, simple and mild procedure for alkylation of phenols, alcohols, amides and acids. *Tetrahedron* **1979**, *35*, 2169−2173.
(44) Brown, H. C.; Choi, Y. M.; Narasimhan, S. Selective reductions. 29. A simple technique to achieve an enhanced rate of reduction of representative organic compounds of borane-dimethyl sulfide. *J. Org. Chem.* **1982**, *47*, 3153−3163.
(45) Lavey, B. J.; Janda, K. D. Catalytic antibody mediated hydrolysis of paraoxon. *J. Org. Chem.* **1996**, *61*, 7633−7636.
(46) Thunus, L.; Lapierre, C. L.; Ghys, A. Synthèse et activité pharmacologique des hydrazino-4 pyridinesulfonamides-3. *Ann. Pharm. Fr.* **1979**, *37*, 451−460.
(47) Hepler, J. R.; Gilman, A. G. G proteins. *Trends Biochem. Sci.* **1992**, *17*, 383−387.
(48) Ui, M.; Nogimori, K.; Makoto, T. *Pertussis Toxin*; Academic Press: New York, 1985.
(49) Seamon, K. B.; Padget, W.; Daly, J. W. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3363−3367.
(50) Griese, M.; Griese, S.; Reinhardt, D. Inhibitory effects of pertussis toxin on the cAMP generating system in human mononuclear leucocytes. *Eur. J. Clin. Invest.* **1990**, *20*, 317−322.
(51) Pessa-Morikawa, T.; Mustelin, T.; Andersson, L. C. Functional maturation of human lymphocytes is accompanied by changes in the G-protein pattern. *J. Immunol.* **1990**, *144*, 2690−2695.
(52) Cowburn, R. F.; O'Neill, C.; Ravid, R.; Winblad, B.; Fowler, C. J. Preservation of Gi-protein inhibited adenylyl cyclase activity in the brains of patients with Alzheimer's disease. *Neurosci. Lett.* **1992**, *141*, 16−20.
(53) Feldman, R. D. Insulin-mediated sensitization of adenylyl cyclase activation. *Br. J. Pharmacol.* **1993**, *110*, 1640−1644.
(54) Spiegel, A. M. Signal transduction by guanine nucleotide binding proteins. *Mol. Cell Endocrinol.* **1987**, *49*, 1−16.
(55) Watkins, D. C.; Northup, J. K.; Malbon, C. C. Pertussis toxin treatment in vivo is associated with a decline in G-protein β-subunits. *J. Biol. Chem.* **1989**, *264*, 4186−4194.
(56) Böyum, A. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **1968**, *21* (Suppl. 97), 77−89.
(57) Brodde, O.-E.; Brinkmann, M.; Schemuth, R.; O'Hara, N.; Daul, A. Terbutaline-induced desensitization of human lymphocyte β2-adrenoceptors. *J. Clin. Invest.* **1985**, *76*, 1096−1101.
(58) Corey, S. J.; Rosoff, P. M. Granulocyte-macrophage colony-stimulating factor primes neutrophils by activating a pertussis toxin-sensitive G protein not associated with phosphatidylinositol turnover. *J. Biol. Chem.* **1989**, *264*, 14165−14171.

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