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[^{35}S]GTPγS binding studies of amphiphilic drugs-activated Gi proteins: A caveat

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ABSTRACT

This paper documents a serious problem met during the testing of Gi protein-activating properties of a new series of synthetic compounds by measuring the induced binding of [^{35}S]GTPγS to different subtypes of Gi protein. The problem arose from the strong affinity between [^{35}S]GTPγS and the tested compounds, that are characterized by several (2–4) positive charges and high lipophilicity. Apparently, such affinity yields insoluble, labelled complexes that, also in the absence of Gi protein, are retained on the filters and give rise to false positive results.

A receptor-independent modulation of the heterotrimeric G proteins is an intriguing purpose. A selective, single-subunit modulator represents a suitable means to intervene in the complex intracellular pathways. Direct modulation could be useful in those pathological conditions where a G protein involvement is already demonstrated. Indeed, altered G proteins are involved in several pathologic conditions: mutations in the Gα inhibitory subunit ($G\alpha_i$) codifying genes have been associated with tumours[1–3] and there is increasing evidence for implications in infections, inflammations, neurological and cardiovascular diseases and endocrine disorders.[4,5] Moreover, a hypofunctionality of $G\alpha_i$ in lymphocytes of cephalalgic and fibromialgic patients was demonstrated.[6,7] Among drugs known to modulate G proteins in a receptor-independent manner,[8–10] a novel series of low molecular weight derivatives were found to be able to stimulate the $G\alpha_i$-protein signalling pathway in human lymphocytes and to activate isolated recombinant $G\alpha_i$ proteins.[11,12] Among these derivatives, a 4-aminopiperidine derivative named BC5 is able to modulate cAMP levels in a recombinant system reconstituted with the isoform 1 of $G\alpha_i$ subunit ($G\alpha_{i1}$) and the intracellular fragments of adenylate cyclase.[13] Moreover, to improve screening accuracy and enhance efficacy, and to reduce the toxicity of therapeutics, we proceeded with the reconstitution of the G protein molecules in a phospholipid bi-layer. For this purpose we chose liposomes as the best biodegradable or biocompatible drug carriers.[14] Aiming to improve the potency and selectivity of previously studied compounds and to establish sounder structure–activity relationships, we have continued our research synthesizing and studying the compounds shown in Table 1.

4-Aminopiperidines **1**–**10** were prepared according to the procedure shown in Scheme 1. Commercially available 4-piperidone hydrochloride monohydrate was treated with di-*tert*-butyl dicarbonate and anhydrous NEt_3, then the intermediate **33** was transformed into **34**–**40** by reductive amination[15] with the appropriate alkylamine. After deprotection with HCl or with trifluoroacetic acid (see experimental part in the Supplementary data**) these compounds gave **1**–**7**. 4-Pentadecilamine piperidine (BC5), prepared by the same method,[11] was alkylated with bromoethylamine hydrobromide to obtain **8**. Compounds **9** and **10** were obtained from BC5 and **8**, respectively, in a three-step procedure acylating with *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine to yield **41** and **42**, and deprotecting the Boc- and trifluoroacetyl groups with trifluoroacetic acid and with K_2CO_3, respectively. Compound **11** was synthesized in a similar way (Scheme 2): 1-pentadecylpiperidin-4-ylamine[11] was acylated with *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine and then deprotected. 4,4'-Bipiperidines **12**–**23** were synthesized as shown in Scheme 3, starting from commercially available 4,4′-bipiperidine dihydrochloride which was treated with 10% NaOH, reacted with BOC-ON [2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile], then treated with the suitable bromoalkyl derivatives and NEt_3 as a scavenger, and finally deprotected to give

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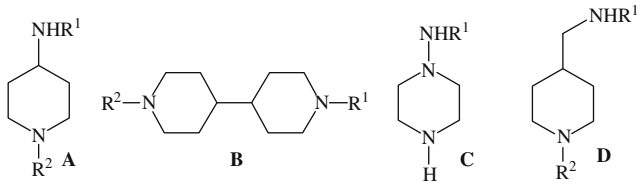
Table 1
Chemical and physical characteristics of final derivatives **1–32**

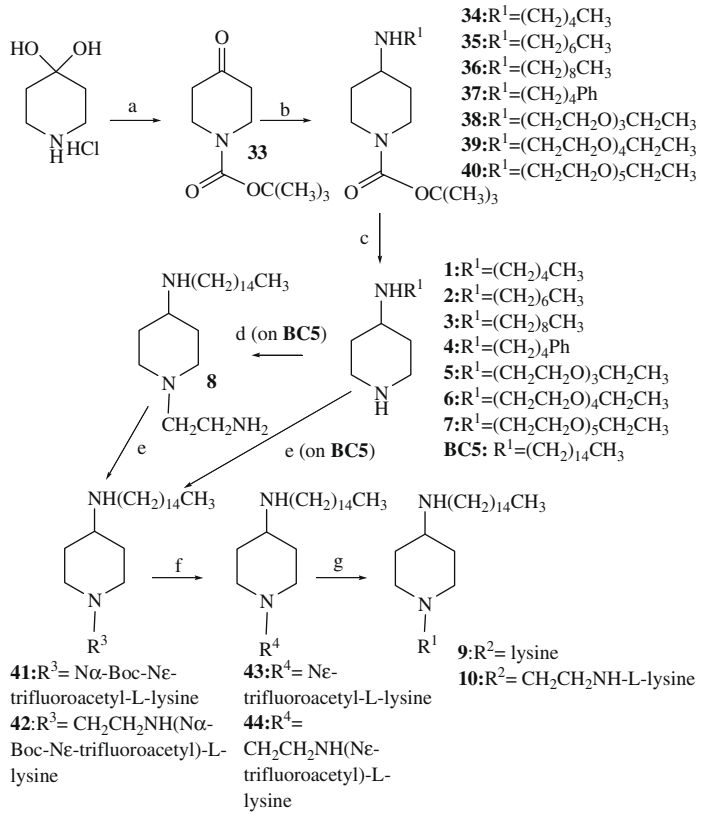


N	Structure	R^1	R^2	Salt (mp °C)[a]	Analysis (salt)
1	A	$(CH_2)_4CH_3$	H	2HCl (309–310)	$C_{10}H_{24}Cl_2N_2$
2	A	$(CH_2)_6CH_3$	H	2HCl (307–308)	$C_{12}H_{28}Cl_2N_2$
3	A	$(CH_2)_8CH_3$	H	2HCl (295–297)	$C_{14}H_{32}Cl_2N_2$
4	A	$(CH_2)_4Ph$	H	2HCl (277–281)	$C_{15}H_{26}Cl_2N_2$
5	A	$(CH_2CH_2O)_3CH_2CH_3$	H	2HCl (low melting)	$C_{13}H_{30}Cl_2N_2O_3$
6	A	$(CH_2CH_2O)_4CH_2CH_3$	H	2HCl (196–200)	$C_{15}H_{34}Cl_2N_2O_4$
7	A	$(CH_2CH_2O)_5CH_2CH_3$	H	2HCl (220–225)	$C_{17}H_{38}Cl_2N_2O_5$
8	A	$CH_2(CH_2)_{13}CH_3$	$CH_2CH_2NH_2$	3HCl (201–205)	$C_{22}H_{50}Cl_3N_3$
9	A	$CH_2(CH_2)_{13}CH_3$	L-Lysine	3HCl (196–200)	$C_{26}H_{57}Cl_3N_4O$
10	A	$CH_2(CH_2)_{13}CH_3$	CH_2CH_2NH-L-lysine	4HCl (202–206)	$C_{28}H_{63}Cl_4N_5O$
11	A	L-Lysine	$CH_2(CH_2)_{13}CH_3$	3HCl (246–249)	$C_{26}H_{57}Cl_3N_4O$
12	B	$(CH_2)_4CH_3$	H	2HCl (269–273)	$C_{15}H_{32}Cl_2N_2$
13	B	$(CH_2)_6CH_3$	H	2HCl (270–271)	$C_{17}H_{36}Cl_2N_2$
14	B	$(CH_2)_8CH_3$	H	2HCl (280–285)	$C_{19}H_{40}Cl_2N_2$
15	B	$(CH_2)_4Ph$	H	2HCl (275–285)	$C_{20}H_{34}Cl_2N_2$
16	B	$(CH_2CH_2O)_3CH_2CH_3$	H	2HCl (low melting)	$C_{18}H_{38}Cl_2N_2O_3$
17	B	$(CH_2CH_2O)_4CH_2CH_3$	H	2HCl (214–218)	$C_{20}H_{42}Cl_2N_2O_4$
18	B	$(CH_2CH_2O)_5CH_2CH_3$	H	2HCl (220–224)	$C_{22}H_{46}Cl_2N_2O_5$
19	B	$CH_2(CH_2)_{13}CH_3$	$CH_2CH_2NH_2$	3HCl (276–280)	$C_{27}H_{58}Cl_3N_3$
20	B	$CO(CH_2)_{13}CH_3$	$CH_2CH_2NH_2$	2HCl (223–225)	$C_{27}H_{55}Cl_2N_3O$
21	B	$CH_2(CH_2)_{13}CH_3$	L-Lysine	3HCl (189–192)	$C_{31}H_{65}Cl_3N_4O$
22	B	$CH_2(CH_2)_{13}CH_3$	CH_2CH_2NH-L-lysine	4HCl (215–218)	$C_{33}H_{71}Cl_4N_5O$
23	B	$CO(CH_2)_{13}CH_3$	L-Lysine	2HCl (237–240)	$C_{31}H_{62}Cl_2N_4O_2$
24	C	$(CH_2)_4CH_3$	—	3HCl (low melting)	$C_9H_{24}Cl_3N_3$
25	C	$(CH_2)_6CH_3$	—	3HCl (238–242)	$C_{11}H_{28}Cl_3N_3$
26	C	$(CH_2)_8CH_3$	—	3HCl (236–240)	$C_{13}H_{32}Cl_3N_3$
27	C	$(CH_2)_4Ph$	—	3HCl (225–230)	$C_{14}H_{26}Cl_3N_3$
28	C	$(CH_2CH_2O)_3CH_2CH_3$	—	3HCl (low melting)	$C_{12}H_{30}Cl_3N_3O_3$
29	D	$(CH_2)_4CH_3$	H	2HCl (low melting)	$C_{11}H_{26}Cl_2N_2$
30	D	$(CH_2)_4Ph$	H	2HCl (220–222)	$C_{16}H_{28}Cl_2N_2$
31	D	$(CH_2CH_2O)_3CH_2CH_3$	H	2HCl (low melting)	$C_{14}H_{32}Cl_2N_2O_3$
32	D	$(CH_2)_{14}CH_3$	CH_2CH_2NHCHO	2HCl (218–220)	$C_{24}H_{51}Cl_2N_3O$

[a] From absolute ethanol/anhydrous diethyl ether.

12–18. In the same manner the already described *N*-pentadecylbipiperidine **55**[11] or *N*-pentadecanoylbipiperidine **56**[11] were obtained; alkylation of these intermediates with bromoethylamine hydrobromide yielded **19** and **20**, while acylation of **55**, **19** and **56** with *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine and subsequent deprotections yielded compounds **21–23**, respectively. Piperazines **24–28** were synthesized as shown in Scheme 4. 1-Amino-4-benzylpiperazine **63**[16] was alkylated with the appropriate bromoalkane or bromoethoxyethane and then debenzylated with $HCOONH_4$ and 10% Pd/C in MeOH to give compounds **24–28**. 4-Methylalkylaminopiperidines **29–32** were prepared as reported in Scheme 5. *N*-benzyl isonipecotic acid **69**[11] was transformed into the corresponding amides **70–72** using ethyl chloroformate, NEt_3 and the appropriate amine. After reduction with borane dimethyl sulphide complex, compounds **73–75** were reduced with $HCOONH_4$ and 10% Pd/C in MeOH to give derivatives **29–31**. *N*-(Piperidin-4-ylmethyl)pentadecan-1-amine **76**,[11] was alkylated with bromoethylamine hydrobromide to obtain compound **32**.

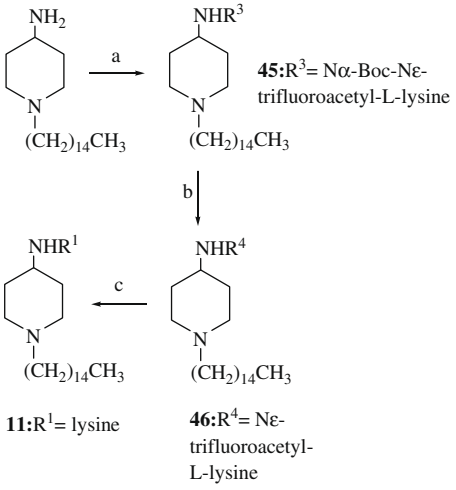
According to previously reported protocols,[11–13] we evaluated the G-protein activation activity of our compounds by measuring the influence of these latter on [^{35}S]GTPγS binding to the different subtypes of Gi protein. These compounds, being lipophilic and positively charged molecules, belong to the class of surface-active drugs. Understandably, the formulation and the screening of surface-active drugs represents a critical issue. It is well known that amphiphilic drugs can self-associate and bind to plasma membrane, causing disruption and solubilization of the lipid bi-layer, similarly to common detergents. As a matter of fact, we were prepared to face the problems related to their tendency to self-assembly that could induce aspecific effects and therefore compromise the reliability of the functional tests of GTPγS binding. However, the cause that eventually aborted our efforts to evaluate the Gi-activating activity of these compounds was unexpected and apparently independent from their tendency to self-aggregate. After a few confusing results, we soon realized that in most cases radioactivity was present on the filters even in the absence of Gi protein, indicating that the molecules tested were able to bind with [^{35}S]GTPγS forming insoluble complexes that were retained on the filters. Of course, this fact rendered the results of the test largely unreliable. In Table 2 some illustrative data obtained both in the presence and absence of Gi protein of selected compounds of the series are reported, in comparison with our standard derivative BC5 (data are reported also for the alphao subunit). Other compounds of the



Scheme 1. Reagents and conditions: (a) $(t\text{BuOCO})_2O$, NEt_3 anhyd; (b) NH_2X, $(i\text{PrO})_4Ti$, $NaBH_3CN$, 25–81% yields; (c) HCl 6 M or CF_3COOH (see experimental part), 67–100% yields; (d) $BrCH_2CH_2NH_2$ HBr, K_2CO_3, 40% yield; (e) *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine, 1,1′-carbonyldiimidazole, 42% yields; (f) CF_3COOH, 83–93% yields; (g) K_2CO_3, 78–96% yields.

series either gave binding values close to basal (no Gi activation; compounds **1–7**, **12–18**, **24–31**) or they behaved like the compounds reported in Table 2 (compounds **8–10**, **21**, **23**).

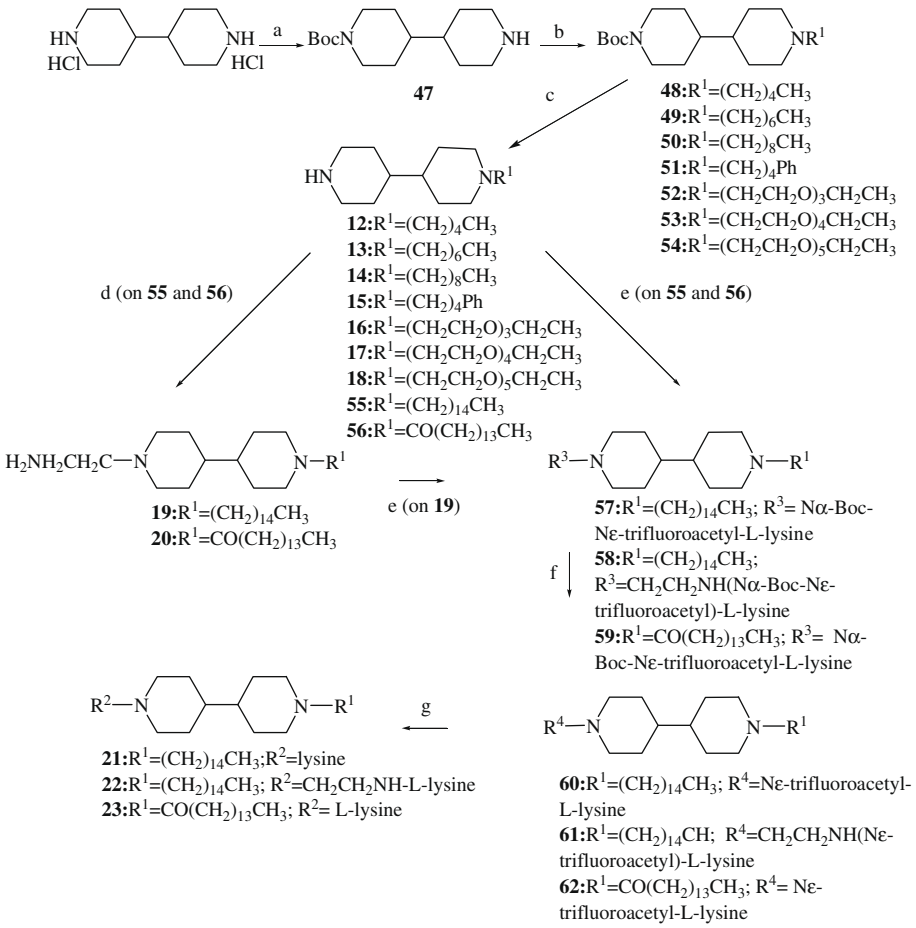
A reasonable explanation for this result is that the positively multi-charged, hydrophobic molecules of our set combine with the negatively charged [^{35}S]GTPγS to give salts that are insoluble in the medium of the assay. The positive correlation between the concentration of the compounds and the radioactivity found on the filter supports this explanation (see for instance compound



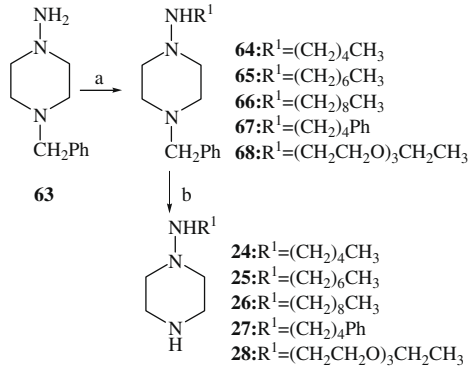
Scheme 2. Reagents and conditions: (a) *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine, 1,1′-carbonyldiimidazole, 76% yield; (b) CF_3COOH, 95% yield; (c) K_2CO_3, 58% yield.

32). On the other hand, the solubility of the salts in the test medium can change according to the structure and dose of tested compounds; this may explain why in many cases (BC5 in Table 2 and all the compounds reported previously)[11,12] we either did not notice the problem or were able to overcome it at suitable doses.

It must be emphasized that this case is somehow different from the micelle aggregation proposed in the recent literature[17,18] to explain 'frequent hits' and 'promiscuous inhibitors' during high throughput screening, that is, the formation of micelle aggregates that interact unselectively with biologically active molecules inhibiting them. However, micelle aggregates could play a role even in this case, contributing to the poor solubility of the salt between the tested compound and [^{35}S]GTPγS. To clarify this point, we checked the tendency of reference compound BC5, and **8** and **22** to give micelle aggregates by monitoring the changes produced on air/water surface tension. As can be seen in Figure 1, the compounds show a critical micelle concentration (cmc) around 1×10^{-4} M (1.0×10^{-4}, 1.41×10^{-4} and 1.44×10^{-4}, respectively). These values suggest that, at lower concentrations, self-aggregation does not critically contribute to the insolubility of the complex with [^{35}S]GTPγS allowing, in the absence of other interferences, a reliable evaluation of their binding to the protein. It was expected that, at cmc or higher concentrations, the micelle formation would aggravate the problem contributing to the failure of the assay: this indeed proved to be the case, as verified experimentally for BC5 (see Table 2). Therefore, the anomalous behaviour found for the compounds reported in Table 2 seems to be due exclusively to the formation of insoluble salts between [^{35}S]GTPγS and some of the compounds tested.

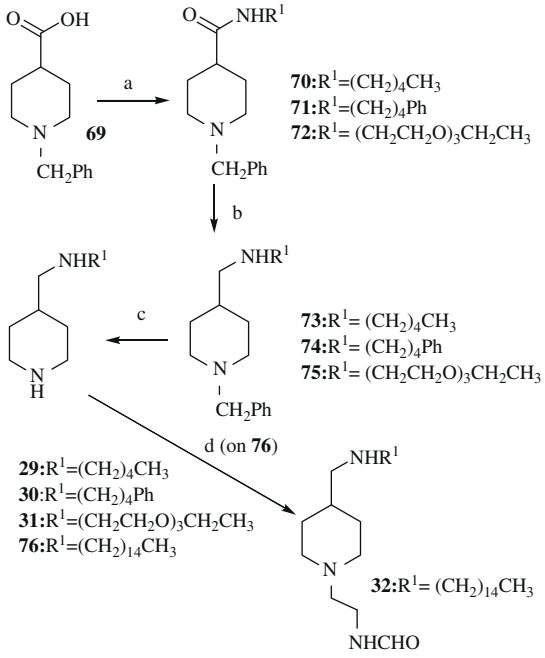


Scheme 3. Reagents and conditions: (a) 10% NaOH, BOC-ON; (b) bromoalkane or bromo-ethoxyethane, NEt_3, 10–47% yields; (c) HCl 6 M or CF_3COOH (see experimental part), 71–100% yields; (d) $BrCH_2CH_2NH_2$ HBr, K_2CO_3, 32–34% yields; (e) *N*α-Boc-*N*ε-trifluoroacetyl-L-lysine, 1,1′-carbonyldiimidazole, 16–93% yields; (f) CF_3COOH, 76–93% yields; (g) K_2CO_3, 60–70% yields.



Scheme 4. Reagents and conditions: (a) BrX, NEt_3, 36–85% yields; (b) Pd/C/$HCOONH_4$, 54–75% yields.

Obviously, as far as Gi activation properties of our compounds are concerned, we can only say that the compounds showing [^{35}S]GTPγS binding close to basal are inactive (see the list reported above). Nothing can be inferred about the activating properties of the compounds listed in Table 2 and of those that behave similarly. A more reliable assay is necessary to evaluate their activity. Our results suggest that much care should be taken when performing binding studies with charged, highly lipophilic molecules.



Scheme 5. Reagents and conditions: (a) ClCOOEt, NH_2R, 39–68% yields; (b) $(CH_3)_2SBH_3$, 36–44% yields; (c) Pd/C/$HCOONH_4$, 24–60% yields; (d) $BrCH_2CH_2NH_2$ HBr, DMF, 40% yield.

Table 2
GTPγS binding on the alphai1 subunit of G proteins (fmol bound in 15 min at 30 °C)

Basal		Basal αo 82.8 ± 4.75 αi1 8.9 ± 0.9	1×10^{-4} M	5×10^{-5} M	1×10^{-5} M	5×10^{-6} M	1×10^{-6} M	Variation[a] (%)
11	With Gi protein			184.8 ± 20.2*	157.0 ± 14.7*	102.8 ± 9.8*	23.7 ± 5.6*	1664
	Without protein			301.9 ± 35.2	234.4 ± 22.2	176.6 ± 19.8	34.6 ± 4.8	
19	With Gi protein			2304.0 ± 235.9*	234.4 ± 19.0*	78.7 ± 13.5*	11.6 ± 1.2	2553
	Without protein			567.4 ± 79.1	149.4 ± 22.8			
20	With Gi protein			102.9 ± 10.9*	87.0 ± 9.8*	76.2 ± 13.7*	30.3 ± 11.1*	877
	Without protein			145.8 ± 9.4	119.8 ± 10.8	107.2 ± 16.5	58.3 ± 12.5	
22	With Gi protein			781.0 ± 97.9*	275.1 ± 22.1*	33.3 ± 9.7*	19.9 ± 9.6	2991
	Without protein			835.8 ± 101.7	192.8 ± 13.2	111.7 ± 9.2		
32	With Gi protein			980.0 ± 90.1*	112.8 ± 16.8*	69.1 ± 6.9*	8.9 ± 2.5	1167
	Without protein			901.2 ± 78.8	321.4 ± 24.6	133.4 ± 12.2		
BC5	With Gi protein		194.8 ± 14.1	195.5 ± 10.2*	70.6 ± 5.2*	49.5 ± 5.6*	15.4 ± 2.6*	693
	Without protein		178.0 ± 10.1	46.7 ± 12.7	28.6 ± 3.7	8.9 ± 2.1	1.2 ± 0.4	
BC5[b]	With Gi protein		1422.5 ± 152	1395.0 ± 94.7*	636.3 ± 78.6*	362.8 ± 58*	102.3 ± 10.3	668
	Without protein		1387.0 ± 178	319.0 ± 18.1	248.8 ± 20.1	68.8 ± 8.1	9.2 ± 2.3	

[a] Variation % in respect to basal at 1×10^{-5} M.
[b] GTPγS binding on the alphao subunit of G proteins, with and without protein.
* $P < 0.05$.

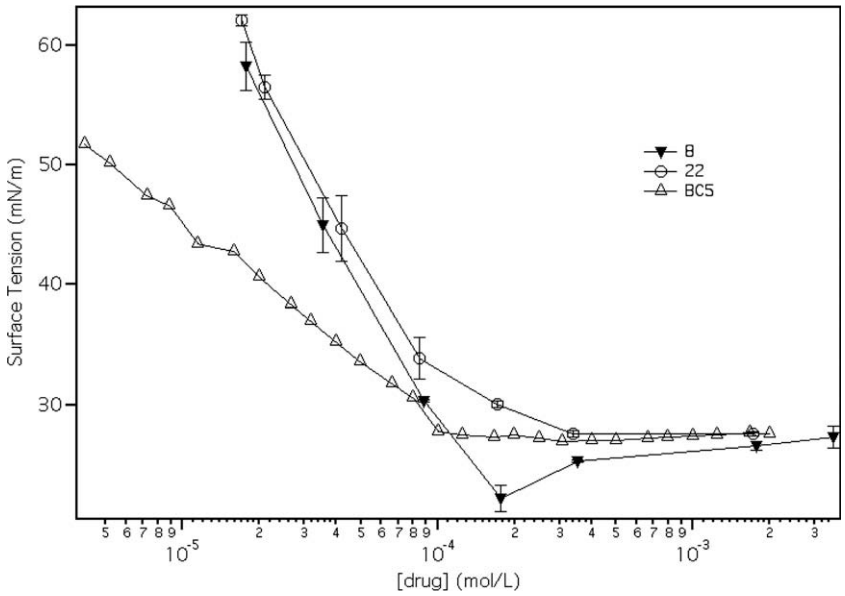


Figure 1. Air/water surface tension measurements.

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Supplementary data

Supplementary data relative to the pharmacological method, chemistry, yield and chromatographic eluent of intermediates, chemical characterization of intermediates and final derivatives and elemental analysis of compounds **1**–**32**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.097.

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