2-Amino-3-aroyl-4,5-alkylthiophenes: Agonist Allosteric Enhancers at Human A_1 Adenosine Receptors

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2-Amino-3-benzoylthiophenes are allosteric enhancers (AE) of agonist activity at the A_1 adenosine receptor. The present report describes syntheses and assays of the AE activity at the human A_1AR (hA_1AR) of a panel of compounds consisting of nine 2-amino-3-aroylthiophenes (**3a**−**i**), eight 2-amino-3-benzoyl-4,5-dimethylthiophenes (**12a**−**h**), three 3-aroyl-2-carboxy-4,5-dimethylthiophenes (**15a**−**c**), 10 2-amino-3-benzoyl-5,6-dihydro-4*H*-cyclopenta[*b*]thiophenes (**17a**−**j**), 14 2-amino-3-benzoyl-4,5,6,7-tetrahydrobenzo[*b*]thiophenes (**18a**−**n**), and 15 2-amino-3-benzoyl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophenes (**19a**−**o**). An in vitro assay employing the A_1AR agonist [^{125}I]ABA and membranes from CHO-K1 cells stably expressing the hA_1AR measured, as an index of AE activity, the ability of a candidate AE to stabilize the agonist-A_1AR-G protein ternary complex. Compounds **3a**−**i** had little or no AE activity, and compounds **12a**−**h** had only modest activity, evidence that AE activity depended absolutely on the presence of at least a methyl group at C-4 and C-5. Compounds **17a**−**c** lacked AE activity, suggesting the 2-amino group is essential. Polymethylene bridges linked thiophene C-4 and C-5 of compounds **17a**−**j**, **18a**−**n**, and **19a**−**o**. AE activity increased with the size of the -$(CH_2)_n$- bridge, $n = 3 < n = 4 < n = 5$. The 3-carbethoxy substituents of **17a**, **18a**, and **19a** did not support AE activity, but a 3-aroyl group did. Bulky (or hydrophobic) substituents at the meta and para positions of the 3-benzoyl group and also 3-naphthoyl groups greatly enhanced activity. Thus, the hA_1AR contains an allosteric binding site able to accommodate 3-aroyl substituents that are bulky and/or hydrophobic but not necessarily planar. A second region in the allosteric binding site interacts constructively with alkyl substituents at thiophene C-4 and/or C-5.

An allosteric enhancer (AE) is a compound that binds to a receptor at a site different from the ligand binding or orthosteric site. Such drugs amplify the activity of an agonist or antagonist wherever and whenever the ligand occupies its receptor. Since the actions of AEs depend on the presence of the natural ligand, they are event- and tissue-specific. The benzodiazepines, which act at GABA receptors, and dihydropyridine calcium channel blockers are familiar examples of drugs that act at allosteric sites.

In 1990 Bruns et al.[1] discovered that 2-amino-3-benzoylthiophenes were weak antagonists but also agonist AEs at the A_1 adenosine receptor (A_1AR) but not at the A_{2A} adenosine receptor (A_{2A}AR). Structure−activity correlations[2] indicated that activity depended on the 2-amino group and the carbonyl oxygen of the benzoyl moiety, suggesting that an intramolecular hydrogen bond making the two aromatic residues coplanar contributed to activity. Certain electron-withdrawing substituents such as chloro or trifluoromethyl at either the meta or para position of the benzoyl moiety greatly augmented activity. Alkyl or aryl groups at thiophene C-4, but not at C-5, promoted activity, as did polymethylene or polymethylenamino bridges linking C-4 and C-5. The most potent compound identified by Bruns was (2-amino-4,5-dimethyl-3-thienyl)[(3-trifluoromethyl)phenyl]methanone, PD81,723, which is compound **12f** in this report. Bruns was not able to further refine the structure−activity rules because the panel of thiophenes available to him was limited.

Allosteric enhancement of A_1AR-mediated responses might have therapeutic potential. Adenosine, acting through A_1ARs, impedes propagation of the cardiac impulse through the atrioventricular node, the "negative dromotropic" effect exploited in the treatment of supraventricular tachyarrythmias with adenosine.[3,4] Several groups have shown that **12f** potentiates the negative dromotropic effect of both exogenous adenosine and of endogenous adenosine released during hypoxia.[5−10] Thus, AEs acting at the A_1AR might be useful in the prophylaxis of paroxysmal supraventricular tachyarrythmias. Similarly, **12f** potentiates the "preconditioning" effect of adenosine, thereby reducing the size of the myocardial infarct produced by a coronary occlusion.[11] Accordingly, AEs for the A_1AR might be useful in the

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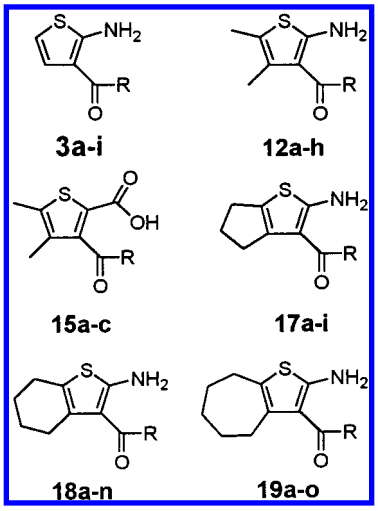
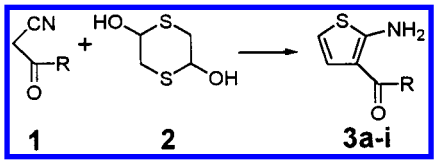


Figure 1. Generic structures of the six panels of thiophenes included in this study.

Scheme 1



prophylaxis of coronary artery disease. Despite these in vivo activities, the 2-aminothiophenes are not ideal drug candidates. First, they are aromatic amines and thus have carcinogenic potential. Second, they tend to be unstable, though perhaps formulation could overcome that drawback. The purpose of the present study was not to develop aminothiophenes as drugs but, rather, to better define their structure−activity rules in order to design new, safer, and more stable AEs.

The present study aims at extending the work of Bruns by examining the AE activity, at a cloned and expressed human A_1AR, of six groups of thiophenes designed to describe those rules in further detail (Figure 1). In terms of the C-4 and C-5 substituents, those groups are as follows: (a) no C-4 or C-5 substituents (**3a**−**i**), (b) 4,5-dimethylthiophenes (**12a**−**h**), and three groups of cycloalkyl[*b*]thiophenes having polymethylene bridges between C-4 and C-5 consisting of (c) three (**17a**−**j**), (d) four (**18a**−**n**), or (e) five (**19a**−**o**) carbon atoms. Within each group, variations in the 3-substituent defined the structure−activity rules governing the contribution of this substituent to AE activity. The sixth group consisted of thiophene-2-carboxylic acids **15a**−**c**, included to test the hypothesis that the 2-amino group is essential for AE activity.

Chemistry

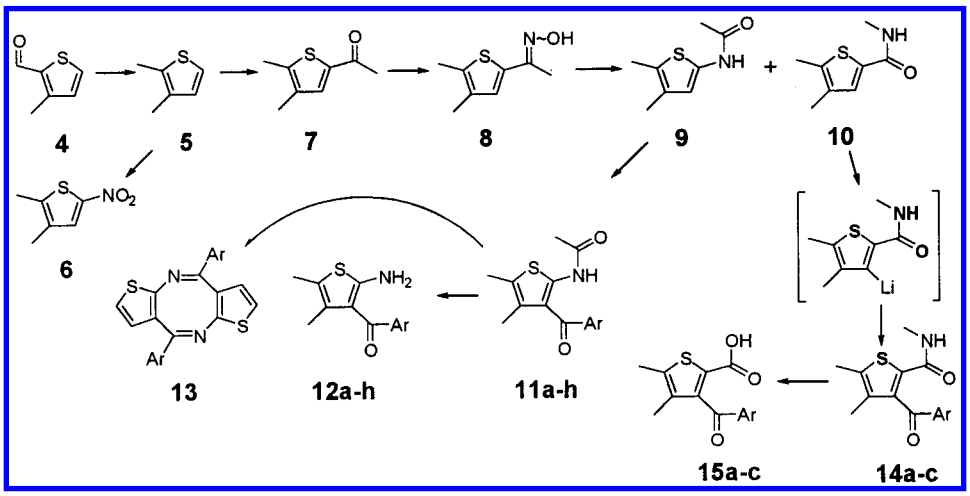
The reaction[12] of 2,5-dihydroxy-1,4-dithiane (thioacetaldehyde dimer, **2**) with an aroylacetonitrile gave 2-amino-3-aroylthiophenes **3a**−**i** (Scheme 1).

The base-catalyzed condensation of an aryl *β*-ketonitrile with 2-butanone to form a mixture of the *E*- and *Z*-isomers of 2-benzoyl-3-ethylcrotonitrile, followed by cyclization with sulfur, is a general method for the synthesis of 2-amino-3-aroyl-4,5-dimethylthiophenes.[13] However, because only the *E*-isomer can react with sulfur, low yields are an inherent disadvantage of that approach. We therefore developed a more efficient alternative synthesis proceeding from 3-methyl-2-thiophenecarboxaldehyde, **4**, to generate 2-amino-3-benzoyl-4,5-dimethylthiophenes **12a**−**h** (Scheme 2). The Huang−Minlon modification[14] of the Wolf−Kishner reduction of **4** gave 2,3-dimethylthiophene, **5**. The original plan called for introducing an amino function at C-2 by nitration of **5** and then reduction of the 4,5-dimethyl-2-nitrothiophene, **6**. Unfortunately, **6** proved difficult to purify, and the subsequent reduction gave a tar. Such an outcome is not surprising, since the instability of 2-amino-4,5-dialkylthiophenes is well known.[15] The alternative synthesis consisted of the tin(IV) chloride-catalyzed Friedel−Crafts acylation of **5** with acetyl chloride to yield 2-acetyl-4,5-dimethylthiophene, **7**. The oxime, **8**, was formed, and PCl_5-catalyzed Beckmann rearrangement of that oxime gave a mixture of 2-acetamido-4,5-dimethylthiophene, **9**, a key intermediate for the synthesis of **12a**−**h**, as well as *N*-methyl 2-carboxamido-4,5-dimethylthiophene, **10**. Friedel−Crafts acylation of **9** by benzoyl chlorides gave 2-acetamido-3-benzoylthiophenes **11a**−**h**. Solvent importantly affected yield; in the case of acylation with benzoyl chloride, replacing benzene with 1,2-dichloroethane improved the yield from 47% to 82%. Base-catalyzed deprotection of **11a**−**h** gave the target thiophenes, **12a**−**h**. Deprotection with acid catalyzed the formation of dimers such as **13** that lacked AE activity. The dimerization was not readily apparent in NMR spectra, but it was evident in high-resolution mass spectrometry. Deprotection of the 2,4,6-trimethylbenzoyl compound **11h** with acid did not lead to dimerization, perhaps a result of the electronic or steric effects of the three methyl groups.

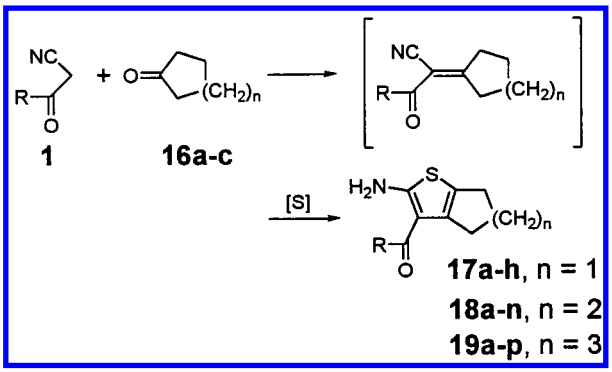
Compound **10** is a side product in the pathway leading to **12a**−**h**, but its derivatives offered the chance to test another inference from the Bruns study, namely, that the 2-amino group is important for activity. That study found that, with the exception of the 2-iodoacetamide, derivatization of the 2-amino group destroyed AE activity. The present study built on that observation by replacing the 2-amino group with a carboxyl group, prepared by the hydrolysis of the amide group of **10**. Friedel−Crafts acylation at C-3 failed, probably because the electron-withdrawing effect of the 2-substituent made the thiophene resistant to acylation. However, lithiation of **10** permitted acylation with benzoyl chlorides, forming the amides, **14a**−**c**. Alkaline hydrolysis then gave compounds **15 a**−**c**.

The method of Gewald[13] served for the syntheses of 5,6-dihydro-4*H*-cyclopenta[*b*]thiophenes **17a**−**j**, 4,5,6,7-tetrahydrobenzo[*b*]thiophenes **18a**−**n**, and 5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophenes **19a**−**o** (Scheme 3). That method consists of the base-catalyzed condensation (Knoevenagel) of a cycloalkanone **16a**−**c** with an aryl *β*-ketonitrile to form an olefin. Subsequently, that olefin undergoes cyclization with sulfur to form a 2-amino-3-aroylthiophene. Most of the present syntheses followed the "one pot" variant, which consists of adding all the reactants and catalyst at once, thereby avoiding the necessity of isolating the intermediate olefin before the reaction with sulfur. The two-step variant served for making multigram quantities of **20l** and **20n**. Diethylamine was usually the catalyst; however, a solid-phase catalyst[16] gave results similar to those using diethyl-

Scheme 2



Scheme 3



amine. Neither LiCl,[17] nor zeolites,[18] which suffice for Knoevenagel condensations of aldehydes, catalyzed the condensation of cycloalkanones **16a**−**c**.

Bromoacetylarenes were the starting materials for the preparation of the β-ketonitriles used to synthesize the thiophenes. Since only a few were commercially available, we prepared them by reacting acetoarenes with elemental bromine in glacial acetic acid,[19] 1,4-dioxane dibromide,[20] copper(II) bromide,[21] or tetrabutylammonium tribromide.[22] Brominations by means of Cu(II)Br or tetrabutylammonium tribromide were rapid, clean, and nearly quantitative. By contrast, brominations with either Br_2/acetic acid or dioxane dibromide required over 2 equiv of brominating agent to drive the reaction to completion. Reacting the bromoacetylarenes with NaCN in cooled ethanol−water generated the β-ketonitriles.[23]

Results and Discussion

Table 1 lists the chemical characteristics of the novel compounds.

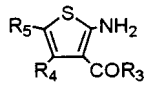
Five panels of compounds provided information about the structure−activity rules governing modifications at thiophene C-3, C-4, and C-5 (Table 2 and Figure 2). The very low to absent activities of **3a**−**i** are evidence that substituents at C-4 and/or C-5 are essential for activity. Consistent with that interpretation, the 4,5-dimethylthiophenes **12a**−**h** have somewhat better AE activity. The AE activities of those cycloalkylthiophenes **17**−**19** having the same C-3 substituents as **12a**−**h** were much higher, evidence that while the dimethyl substituents could support activity, their contribution was less than optimal. In the study by Bruns,[1] AE activity increased in proportion to the size of the C4−C5 polymethylene bridge, $-(CH_2)_3- < -(CH_2)_4- < -(CH_2)_5-$. However, that comparison was limited to three compounds. Between-group comparisons of members of **17**, **18**, and **19** having the same C-3 substituents showed that AE activity increased according to the size of the C4−C5 polymethylene bridge (Figure 2).

The 3-aroyl moieties contributed importantly to AE activity. None of the cycloalkylthiophenes having a 3-carboxyethyl substituent, namely, **17a**, **18a**, and **19a**, were active. An unsubstituted benzoyl group supported a low level of AE activity, and both 3- and 4-fluorobenzoyl groups generally did likewise. Other benzoyl substituents increased AE activity, the rank order for all substituents being H = F ≪ Cl < Br < I = Ph = cHex. Both the 1- and the 2-isomers of 3-naphthoylthiophenes had substantial AE activity. QSAR analysis[24] showed that neither of the electronic parameters, σ_m or σ_p, of the 3-benzoyl substituent accounted for differences in AE activity (r^2 for the regressions of AE activity on either Hammett parameter were <0.1 and were not significant; data not shown). However, the hydrophobic and steric parameters, π and molar refractivity, respectively, better accounted for the effect of the 3-aroyl substituents on AE activity (Figure 3). That the analysis could not distinguish between hydrophobicity and steric bulk is not surprising, since those substituent parameters tend to be covariant.[24] For the substituent groups studied here, the regression of π on molar refractivity had $r^2 = 0.83$. Although most of the 3-aroyl substituents were planar, thiophenes having 4-phenylbenzoyl (**17i**, **18k**, **19m**) or 4-cyclohexylbenzoyl (**18l**, **19n**) substituents had excellent activity. Because the phenyl group can rotate around the axis of the bond joining it to the benzoyl moiety and a cyclohexane group is not planar, the high AE activity of those compounds suggests that planarity in that portion of the molecule is not critical.

Thiophene-2-carboxylic acids **17a**−**c** lacked activity, support for the idea[2] that AE activity depends on the 2-amino group.

In summary, analysis of the results by QSAR supports a model of the hA_1AR that contains an allosteric binding site able to accommodate 3-aroyl substituents that are bulky and hydrophobic but not necessarily planar. A second region in the A_1AR interacts additively with alkyl substituents at thiophene C-4 and/or C-5. The lack

Table 1. Characteristics of Novel 2-Aminothiophenes



no.	R_3, R_4, R_5	yield, %	purification[a]	mp, °C	formula	anal.
3b	3-FPh, H, H	47	E	155−6	$C_{11}H_8FNOS$	C, H, N
3d	3-BrPh, H, H	56	E	135	$C_{11}H_8BrNOS$	C, H, N
3e	4-FPh, H, H	69	E	142−4	$C_{11}H_8FNOS$	C, H, N
3g	4-BrPh, H, H	41	E	153−4	$C_{11}H_8BrNOS$	C, H, N
3h	3,4-Cl_2Ph, H, H	71	E	138−40	$C_{11}H_7Cl_2NOS$	C, H, N
3i	2-naphth, H, H	63	E	143−5	$C_{15}H_{11}NOS$	C, H, N
12b	3-FPh, Me, Me	54	E	106	$C_{13}H_{12}FNOS$	C, H, N
12c	3-ClPh, Me, Me	38	E	114−7	$C_{13}H_{12}ClNOS$	C, H, N
12d	3-BrPh, Me, Me	94	E	128−30	$C_{13}H_{12}BrNOS$	C, H, N
12e	3-CH_3Ph, Me, Me	63	E	118−20	$C_{14}H_{15}NOS$	C, H, N
12g	3-PhPh, Me, Me	93	E	161−3	$C_{19}H_{17}NOS$	C, H, N
12h	mesityl, Me, Me	84	E	156−9	$C_{16}H_{19}NOS$	C, H, N
15a	Ph, 2-COOH	71	E	212−4	$C_{13}H_{13}OS$	C, H, N
15b	3-CF_3Ph, 2-COOH	90	E	187−9	$C_{15}H_{11}F_3O_3S$	C, H, N
15c	4-PhPh, 2-COOH	86	E	218−19	$C_{20}H_{16}O_3S$	C, H, N
17a	CO_2Et, -$(CH_2)_3$-	88	E	95−7	$C_{10}H_{13}NO_2S$	C, H, N
17c	3ClPh, -$(CH_2)_3$-	59	E	185	$C_{14}H_{12}ClNOS$	C, H, N
17d	3BrPh, -$(CH_2)_3$-	52	E	200−3	$C_{14}H_{12}BrNOS$	C, H, N
17e	4F-Ph, -$(CH_2)_3$-	74	E	151−3	$C_{14}H_{12}FNOS$	C, H, N
17i	2-naphth, -$(CH_2)_3$-	55	E	154−6	$C_{15}H_{11}NOS$	C, H, N
18a	CO_2Et, -$(CH_2)_4$-	73	E	95−7	$C_{11}H_{15}NO_2S$	C, H, N
18c	3-FPh, -$(CH_2)_4$-	52	H	112−4	$C_{15}H_{14}FNOS$	C, H, N
18e	3-BrPh, -$(CH_2)_4$-	37	H	118−21	$C_{15}H_{14}BrNOS$	C, H, N
18j	4-CH_3Ph, -$(CH_2)_4$-	79	E	138−40	$C_{16}H_{17}NOS$	C, H, N
18k	4-CNPh, -$(CH_2)_4$-	32	E	208−10	$C_{16}H_{14}N_2OS$	C, H, N
18l	4-PhPh, -$(CH_2)_4$-	14	E	109−11	$C_{21}H_{19}NOS$	C, H, N
18m	4-cHexPh, -$(CH_2)_4$-	23	E	114−6	$C_{21}H_{25}NOS$	C, H, N
18n	2-naphth, -$(CH_2)_4$-	18	H	104−6	$C_{19}H_{17}NOS$	C, H, N
19a	CO_2Et, -$(CH_2)_5$-	75	E	116−9	$C_{12}H_{17}NO_2S$	C, H, N
19c	3ClPh, -$(CH_2)_5$-	21	E	74−6	$C_{16}H_{16}ClNOS$	C, H, N
19d	3-BrPh, -$(CH_2)_5$-	28	E	80−2	$C_{16}H_{16}BrNOS$	C, H, N
19e	3-IPh, -$(CH_2)_5$-	38	E	100−1	$C_{16}H_{16}INOS$	C, H, N
19f	4FPh, -$(CH_2)_5$-	28	H	78−80	$C_{16}H_{16}FNOS$	C, H, N
19g	4ClPh, -$(CH_2)_5$-	33	H	97−9	$C_{16}H_{16}ClNOS$	C, H, N
19h	4BrPh, -$(CH_2)_5$-	26	E	153−5	$C_{16}H_{16}BrNOS$	C, H, N
19i	4-IPh, -$(CH_2)_5$-	64	E	176−8	$C_{16}H_{16}INOS$	C, H, N
19j	3-CH_3OPh, -$(CH_2)_5$-	30	E	71−3	$C_{17}H_{19}NO_2S$	C, H, N
19k	4-CH_3OPh, -$(CH_2)_5$-	84	E	124−6	$C_{17}H_{19}NO_2S$	C, H, N
19l	4-PhPh, -$(CH_2)_5$-	42	E	55−7	$C_{21}H_{21}NOS$	C, H, N
19m	4-cHxPh, -$(CH_2)_5$-	31	E	131−3	$C_{21}H_{27}NOS$	C, H, N
19n	1-naphth, -$(CH_2)_5$-	20	E	92−4	$C_{19}H_{19}NOS$	C, H, N
19o	2-naphth, -$(CH_2)_5$-	34	E	120−2	$C_{19}H_{19}NOS$	C, H, N

[a] Solvents for crystallization: E, ethanol−water; H, hexane.

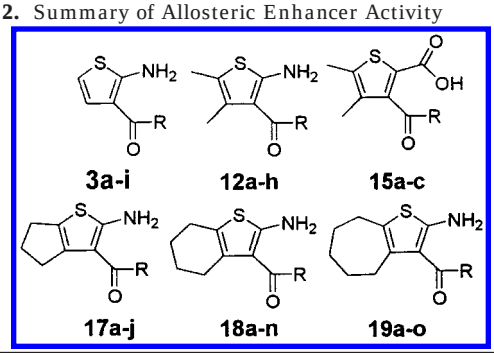
of activity of thiophenes **3a**−**i** and the thiophene-2-carboxylic acids **17a**−**c** suggests that the 2-amino group is necessary but not sufficient for AE activity at the A_1-AR.

The original design of the study included measurements of the A_1AR antagonistic activity of candidate AEs, measured as their ability to compete with the binding of [^{3}H]CPX. Table 2 includes those results. Several of the candidate AEs had substantial antagonistic activity. However, when it became clear part of the way through the study that the two activities were unrelated ($r^2 = 0.057$, $n = 28$), assays of antagonism were discontinued.

All of the compounds underwent screening for AE activity at both the hA_{2A}AR and the hA_3AR, but none were active. Likewise, the AEs did not affect the rate of dissociation of antagonists from the hA_1AR, evidence that there was no allosteric effect on antagonist binding. Differential activity at the A_1AR and A_3AR may have a practical application. Since N^6-substituted adenosines are agonists at both the A_1AR and A_3AR, assigning a biological response to one or the other receptor on the basis of an agonist activity profile may give ambiguous results. Potentiation by an allosteric enhancer could be an additional criterion for deciding that the A_1AR rather than the A_3AR initiates a response.

This study included seven compounds previously studied by Bruns et al.[2] (**12a**, **12f**, **17b**, **18b**, **18d**, **18f**, **19b**), seven by van der Klein et al.[25] (**3a**, **3c**, **18d**, **18f**, **18g**, **18h**, **18i**), and 15 by Baraldi et al.[26] (**3a**, **3f**, **17b**,**f**−**h**, **18b**,**f**−**h**,**l**, and **19b**,**f**,**g**,**k**). The studies of Bruns et al. and of van der Klein et al. assayed A_1AR AE activity under different conditions and examined the rat rather than the human A_1AR. The study of Baraldi et al. employed a functional assay in cultured cells expressing the hA_1AR. Accordingly, direct comparisons of the results of the present study with the earlier studies are unwarranted. However, the data reported by Bruns et al. and van der Klein et al. support the idea that the size/hydrophobicity of the 3-aroyl group influences AE activity as much as the kinds of alkyl groups at C-4 and C-5. The present study identified several compounds substantially more active than **12f**, which was the most active compound identified by Bruns. In that regard our

Table 2. Summary of Allosteric Enhancer Activity



no.	R	AE score, %[a]	inhibition of [^{3}H]CPX binding, %[a]
3a	Ph	0.2 ± 0.01	15 ± 6
3b	3-FPh	0	
3c	3-ClPh	0	
3d	3-BrPh	0.2 ± 0.03	
3e	4-FPh	0	
3f	4-ClPh	0	
3g	4-BrPh	0.8 ± 0.3	
3h	3,4-Cl_2Ph	1.7 ± 0.8	
3i	2-naphth	3.2 ± 1.8	
12a	Ph	9	
12b	3-FPh	0	
12c	3-ClPh	14 ± 1	
12d	3-BrPh	16 ± 4	
12e	3-CH_3Ph	18 ± 0.5	
12f	3-CF_3Ph	19 ± 2.9	42 ± 7
12g	4-PhPh	12 ± 1.9	
12h	mesityl	0	
15a	Ph	0	
15b	3-CF_3Ph	0	
15c	4-PhPh	0	
17a	OEt	0.4 ± 0.1	58 ± 9
17b	Ph	20 ± 3.6	56 ± 7
17c	3-FPh	16 ± 2	
17d	3-ClPh	13 ± 3	56 ± 15
17e	3-BrPh	68 ± 1	36 ± 7
17f	4-FPh	22 ± 4	40 ± 4
17g	4-ClPh	70 ± 7	
17h	4-BrPh	62 ± 5	20 ± 6
17i	4-PhPh	38 ± 3	
17j	2-naphth	31 ± 4	21 ± 3
18a	OEt	3.5 ± 1.8	53 ± 5
18b	Ph	19 ± 5	42 ± 9
18c	3-FPh	22 ± 5	35 ± 4
18d	3-ClPh	70 ± 9	78 ± 8
18e	3-BrPh	49 ± 1	49 ± 13
18f	4-FPh	17 ± 3	56 ± 6
18g	4-ClPh	68 ± 10	69 ± 9
18h	4-BrPh	83 ± 5	61 ± 7
18i	4-IPh	86 ± 13	62 ± 11
18j	4-CH_3Ph	19 ± 7	36 ± 4
18k	4-CNPh	19 ± 0.9	21 ± 6
18l	4-PhPh	33 ± 2	
18m	4-cHxPh	99 ± 6	47 ± 7
18n	2-naphth	86 ± 1	
19a	OEt	0.3 ± 0.01	38 ± 3
19b	Ph	13 ± 3.8	68 ± 6
19c	3-ClPh	65 ± 8	10 ± 2
19d	3-BrPh	78 ± 2.6	7 ± 3
19e	3-IPh	86 ± 9	
19f	4-FPh	22 ± 2.7	
19g	4-ClPh	65 ± 6.7	
19h	4-BrPh	86 ± 3.8	7 ± 4
19i	4-IPh	96 ± 4.7	9 ± 4
19j	3-OCH_3Ph	99 ± 3.1	13 ± 3
19k	4-OCH_3Ph	85 ± 10	
19l	4-PhPh	88 ± 4.6	
19m	4-cHxPh	77 ± 11	
19n	1-naphth	81 ± 7.2	
19o	2-naphth	75 ± 9.6	

[a] Concentration of allosteric enhancer was 100 μM. All data are mean ± SEM of three separate assays, each in triplicate.

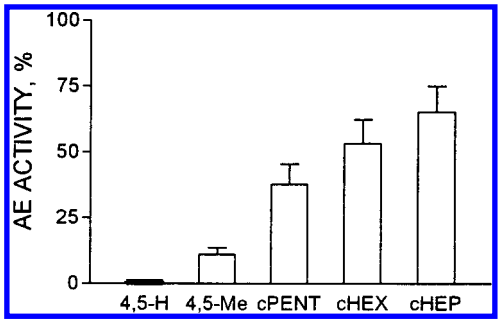


Figure 2. Influence of the size of the thiophene C-4 and C-5 substituents on AE activity. Legend: 4,5-H, compounds **3a**−**i**; 4,5-Me, compounds **12a**−**h**; cPENT, compounds **17b**−**i**; cHEX, compounds **18b**,**d**−**h**,**l**,**m**; and cHEP, compounds **19b**−**d**,**f**−**h**,**l**,**o**. Note that methyl groups are the smallest substituents that support activity and that activity of the cycloalka-[*b*]thiophenes parallels the size of the cycloalkyl moiety.

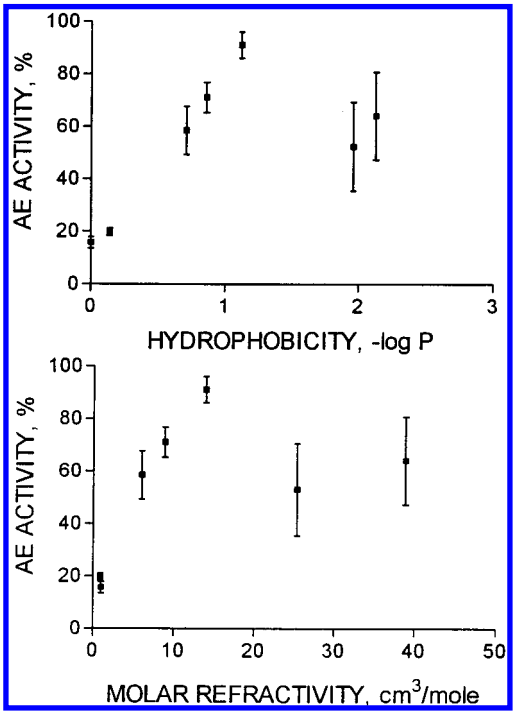


Figure 3. QSAR analysis of the effect of 3-aroyl substituents on the AE activity of 2-amino-3-aroylthiophenes. Top panel: dependence of AE activity on log *P*, an index of hydrophobicity. Bottom panel: dependence of AE activity on molar refractivity, an index of steric bulk. See text for additional discussion.

observations support those of van der Klein et al.[25] but not Baraldi et al., who found that **12f** was the most potent compound tested.[26]

Assay of Allosteric Enhancer Activity. The assay devised to screen candidates for AE activity measures the ability of an AE to stabilize the agonist-receptor-G protein ternary complex,[7,27] manifested as a decrease in the rate of agonist dissociation (Figure 4). The first phase of the assay achieved agonist binding equilibrium. The second phase, a 5 min exposure to a candidate AE, was sufficiently long for binding of the AE to the ternary complex without discernible (<5%) loss of bound agonist due to any coexisting antagonist activity of the AE. The third phase measured the rate at which bound agonist dissociated, driven both by competition at the orthosteric site with a large excess of antagonist as well as by displacement of GDP from the G protein by GTPγS.

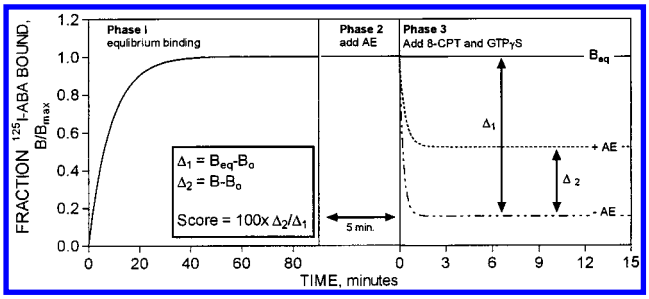


Figure 4. Experimental protocol for the assay of allosteric enhancer activity. See text for discussion.

Adding GTPγ greatly reduced the time necessary to perform an assay. In the absence of GTPγS, dissociation can be very slow, $t_{1/2} \sim 60$ min. Upon addition of GTPγS, a fraction of the receptors release the agonist relatively quickly and the remainder release the agonist very slowly. We have previously shown that AEs increase the fraction of receptors coupled to G proteins, which bind agonists with high affinity. Our data are consistent with the possibility that in kinetic experiments the rapidly dissociating component represents receptors not coupled to G proteins and the slowly dissociating component represents receptors tightly coupled to G proteins. Our data suggest that the population of receptor-G protein complexes increases and remains relatively stable in the presence of enhancers, even after the addition of GTPγS. Over the time course of the assay, dissociation can be very slow, but in such instances it drops toward the level of unspecific binding in a matter of hours.

Experimental Section

Melting points are uncorrected. Elemental analyses agreed within ±0.4% of calculated composition. ^{1}H NMR spectra were consistent with the putative structures. Trans-World Chemicals, Rockville, MD, supplied 3′-iodoacetophenone. One recrystallization from methanol removed minor impurities from 4-acetylbiphenyl. All other starting materials were from Aldrich and were used as received. The brominations[19−22] of acetylarenes and their conversions to aroylacetonitriles[23] followed the methods cited.

(2-Amino-thien-3-yl)(phenyl)methanone (3a) General Method A. Benzoylacetonitrile (1.45 g, 10 mmol), 2,5-dihydroxy-1,4-dithiane (0.76 g, 5 mmol), and diethylamine (0.73 *g* = 1.04 mL, 10 mmol) in 4 mL of absolute ethanol were heated in a Teflon-sealed pressure tube for 4 h at 50 °C with frequent stirring on a vortex mixer. By 2 h starting materials had dissolved, and shortly thereafter the product began to crystallize. After refrigerating the tube overnight, the product was filtered off and washed with a little methanol to give bright yellow crystals. Yield: 1.3 g, 64%. ^{1}H NMR ($CDCl_3$) δ: 6.14 (d, 1H, H-5), 6.88 (d, 1H, H-4), 6.95 (br s, 2H, NH_2), 7.5−7.7 (m, 5H, C$_6H_5$).

2,3-Dimethylthiophene (5). Heating a mixture of 3-methyl-2-thiophene carboxaldehyde, **4** (58.6 g, 464 mmol), 80% hydrazine hydrate (97 mL, 1.62 mol), and 200 mL of ethylene glycol to an internal temperature of 130−160 °C caused hydrazine and water to distil. The reaction mixture was cooled to below 60 °C, and the water-immiscible fraction of the distillate was returned to the flask. The addition of KOH (91.0 g, 1.62 mol) and reheating caused vigorous gas evolution when the temperature reached 90−100 °C. Reflux continued for 15 min after gas evolution ceased; steam distillation then separated **5**. Product in the distillate was extracted into ether, the extract washed with 6 N HCl, dried over $CaCl_2$, and evaporated. Distillation over sodium gave **5** as a colorless oil, bp 139.5−140.5, yield 39.8 g, 77%. ^{1}H NMR ($CDCl_3$) δ: 2.21, s, 3H, CH_3; 2.41, s, 3H, CH_3; 6.84, d, J = 5.1 Hz, 1H, H-4; 7.03, d, J = 5.2 Hz, 1H, H-5. ^{13}C NMR ($CDCl_3$) δ: 13.0, 13.6, 120.6, 129.9, 132.6, 133.0.

(4,5-Dimethyl-2-thienyl)(methyl)methanone (7). A solution of **5** (15.16 g, 135 mmol) and acetyl chloride (9.6 mL, 135 mmol) in 60 mL of benzene dried over Na was cooled to − 5 °C and vigorously stirred during the addition of a solution of tin(IV) chloride in 50 mL of benzene over a period of 1 h. The reaction mixture was removed from the cold bath and stirred for an additional hour at room temperature. The slow addition of 4 mL of concentrated HCl in 28 mL of water quenched the reaction. The organic layer was separated, washed with 2 × 10 mL water, dried over Na_2SO_4, and evaporated to give 20.8 g of crude product. Chromatography on a column of silica eluted with petroleum ether:ethyl acetate (10:1) and evaporation of relevant fractions gave a viscous yellow oil, 16.42 g, 79%. ^{1}H NMR ($CDCl_3$) δ: 2.08, s, 3H, COCH_3; 2.31, s, 3H, CH_3; 2.41, s, 3H, CH_3; 7.33, s, 1H, H-3. ^{13}C NMR ($CDCl_3$) δ: 13.3, 13.7, 26.1, 134.7, 135.3, 139.1, 143.4, 190.0.

1-(4,5-Dimethyl-2-thiophen-2-yl)-ethanone Oxime (8). A mixture of **7** (33.1 g, 215 mmol), hydroxylamine hydrochloride (32.9 g, 473 mmol), and barium carbonate (91.7 g, 495 mmol) in 500 mL of ethanol was heated at reflux for 8 h, the salts were filtered, and the filtrate was evaporated to an off-white solid. Crystallization from ethanol−water afforded 30.8 g (85%) of pure **8**. Four recrystallizations improved the *E*:*Z* ratio of isomers from 4:1 to 14:1. ^{1}H NMR ($CDCl_3$) δ: 2.11 (s, 3H, CH_3C=NOH), 2.26 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 6.94 (s, 1H, H-3), 9.62 (br s, 1H, O*H*). ^{13}C NMR ($CDCl_3$) δ: 11.4, 12.6, 12.9, 129.2, 132.7, 133.1, 135.8, 149.4.

***N*-(4,5-Dimethyl-thiophen-2-yl)acetamide (9) and 4,5-Dimethyl-thiophene-2-carboxylic Acid Methylamide (10).** A solution of **8** (0.304 g, 1.8 mmol) in 5 mL of dry ether was cooled to 0 °C and stirred vigorously during the addition of PCl_5 (0.4 g, 1.9 mmol) at a rate that kept the temperature at 0 °C. Stirring on ice continued for 15 min and at room temperature for an additional 30 min. The addition of 1 mL of water at a rate keeping the temperature at <20 °C quenched the reaction. Under cooling, NaOH was added to bring the pH to 5−6, and the product was extracted into ether. Evaporation gave 0.324 g of crude product that was purified by elution from a silica gel column with petroleum ether−ethyl acetate 1:1 to give **9** (0.097 g, 32%). ^{1}H NMR ($CDCl_3$) δ: 2.03 (s, 3H, COCH_3), 2.16 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 6.39 (s, 1H, H-3), 9.08 (br s, 1H, N*H*C=O). ^{13}C NMR ($CDCl_3$) δ: 12.3, 13.4, 23.0, 115.6, 124.8, 129.5, 134.2, 167.3. Additional fractions contained **10** (0.060 g, 20%). ^{1}H NMR ($CDCl_3$) δ: 2.10 (s, 3H, C$_H$3), 2.33 (s, 3H, C*H*3), 2.94 (d, 3H, NHC*H*3), 6.21 (br s, 1H, N*H*, 7.22 (1H, Ar*H*). ^{13}C NMR ($CDCl_3$) δ: 13.4, 13.5, 26.6, 131.0, 133.2, 134.0, 138.2, 162.8.

***N*-(3-Benzoyl-4,5-dimethyl-thiophen-2-yl)acetamide (11a). General Method B.** A solution of 1.71 M tin(IV) chloride (3.1 mL, 5.3 mmol) in 1,2-dichloroethane was added dropwise to a suspension of **9** (0.241 g, 1.42 mmol) and benzoyl chloride (0.31 mL, 2.66 mmol) in 1,2-dichloroethane, and the mixture was refluxed for 10.5 h. The reaction was quenched with ice, and the organic phase was washed sequentially with 2 N HCl, water, and 2 N NaOH. Drying over $CaCl_2$ and evaporation gave a solid that was purified by chromatography on silica gel eluted with petroleum ether−ethyl acetate 5:1. Recrystallization from ethanol−water gave 0.32 g of pure product as yellow crystals, 82%. ^{1}H NMR ($CDCl_3$) δ: 1.6 (s, 3H, CH_3), 2.23 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 7.4−7.6 (m, 5H, C$_6H_5$), 11.1 (br s, 1H, N*H*). ^{13}C NMR ($CDCl_3$) δ: 12.4, 14.8, 23.6, 122.4, 124.7, 127.6, 128.3, 128.4, 131.9, 140.3, 146.4, 167.4, 195.0.

(2-Amino-4,5-dimethyl-thiophen-3-yl)(phenyl)methanone (12a). General Method C. A solution of **11a** (0.3 g, 1.1 mmol) in KOH (3.5 equiv in methanol−water 1:1) was refluxed for 45 min, evaporated, and taken up in dichloromethane. The solution was washed three times with water, dried, and evaporated to a solid that was recrystallized from ethanol−water as yellow crystals. Yield: 0.25 g, 98%. ^{1}H NMR ($CDCl_3$) δ: 1.5 (s, 3H, CH_3), 2.1 (s, 3H, CH_3), 6.4 (br s, 2H, NH_2), 7.2−

7.5 (m, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$) δ: 12.5, 15.2, 114.9, 117.2, 127.8, 128.0, 128.8, 130.4, 141.7, 162.8, 193.0.

4,9-Bis-(3-fluorophenyl)-2,3,7,8-tetramethyl-[2,3-*b*:2′,3′-*b*′]-dithieno-1,5-diazocine (13). A solution of **11a** (0.54 g, 1.86 mmol) in ethanolic 0.5 N HCl was heated at reflux for 7 h, cooled, and alkalinized with NaOH. Extracting into dichloromethane, drying, and evaporation gave a solid that was purified by chromatography on silica gel eluted with petroleum ether−ethyl acetate 10:1. Crystallization from ethanol−water gave orange crystals, 0.254 g, 57%. ^{1}H NMR ($CDCl_3$) δ: 1.6 (s, 3H, CH_3), 2.3 (s, 3H, CH_3), 7.1−7.5 (m, 4H, C_6H_4F). ^{13}C NMR ($CDCl_3$) δ: 13.0, 13.2, 115.4 (d, J = 22.8 Hz), 118.1 (d, J = 21.3 Hz), 123.5, 124.8 (d, J = 2.6 Hz), 130.3, 130.7, 140.2 (d, J = 7.3 Hz), 153.0, 162.8 (d, j = 246.2 Hz), 169.1 (d, J = 2.6 Hz). ES-MS m/z 463.1 (M + 1), 485.1 (M + Na).

(4,5-Dimethyl-2-methylcarbamoyl-thiophen-3-yl)(phenyl)methanone (14a). A solution of **10** (0.40 g, 2.37 mmol) in 20 mL of dry THF was cooled to −70 °C and stirred during the addition of *tert*-butyllithium (5.21 mmol). After 30 min of stirring, benzoyl chloride (0.42 g = 0.35 mL, 3 mmol) was added, and the mixture was warmed to room temperature. Workup consisted of quenching the reaction with saturated aqueous NH_4Cl and extraction of product into ethyl acetate. The extract was dried over $MgSO_4$ and evaporated, and the product was purified by chromatography on silica gel eluted with hexanes−ethyl acetate 1:1. Yield: 0.356 g, 55%. ^{1}H NMR ($CDCl_3$) δ: 1.83 (s, 3H, CH_3), 2.37 (s, 3H, CH_3), 2.79 (d, 3H, NHCH_3), 6.58 (br s, 1H, NH), 7.42−7.78 (m, 5H, ArH).

(2-Carboxy-4,5-dimethylthiophen-3-yl)(phenyl)methanone (15a). A solution of **14a** (0.281 g, 1.03 mmol) in methanol−water 1:1 containing 10% KOH was heated at reflux for 12 h, neutralized, and extracted with ethyl acetate. The solid after evaporation was crystallized from ethanol. Yield: 0.19 g, 71%.

(2-Amino-4,5-dihydrocyclopenta[*b*]thiophen-3-yl)(3-bromophenyl)methanone (17d). General Method D. A mixture of sulfur (0.176 g, 5.5 mg-at), 3-bromobenzoylacetonitrile (1.35 g, 5.5 mmol), and cyclopentanone (0.463 g = 0.482 mL, 5.5 mmol) in 4 mL anhydrous ethanol was heated at 50 °C in a Teflon-capped pressure tube for 4 h. Cooling overnight deposited crystalline product, which was filtered off, washed with a little cold methanol, and dried. TLC showed the material was pure; yield 1.2 g, 62%. ^{1}H NMR ($CDCl_3$) δ: 2.16 (m, 4H, H-4 and H-6), 2.65 (m, 2H, H-5), 7.07 (br s, 2H, NH_2), 7.3−7.6 (m, 4H, C_6H_4Br).

(2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(4-biphenyl)methanone (18l). A mixture of 4-phenylbenzoylacetonitrile (4.42 g, 0.02 mol), cyclohexanone (1.96 g = 2.1 mL, 0.02 mol), β-alanine (0.18 g, 0.002 mol), glacial acetic acid (2 mL), and toluene (100 mL) was heated at reflux in a flask fitted with a Dean−Stark trap and condenser. After 18 h, TLC (hexane:ethyl acetate 3:1) showed complete conversion of the nitrile, R_f 0.48, to the olefin, R_f 0.67. The residue after evaporation was taken up in ethyl acetate, washed twice with 50 mL of water, dried over $MgSO_4$, and evaporated to a glass. Weight: 4.6 g, 76%. Sulfur (0.673 g, 0.021 mol) was suspended in a solution of the olefin in 50 mL of anhydrous ethanol, diethylamine (1 mL) was added, and the dark suspension was stirred at room temperature until the sulfur had disappeared. Product that crystallized out on cooling in an ice bath was filtered off, washed with a little methanol, and dried. TLC (hexane:ethyl acetate 1:3) showed only product, R_f 0.50. Yield: 4.5 g, 67% based on starting nitrile. ^{1}H NMR δ: 1.57 (m, 2H, cyclohexyl), 1.81 (m, 2H, cyclohexyl), 1.97 (q, 2H, cyclohexyl), 2.59 (q, 2H, cyclohexyl), 6.75 (br s, 2H, NH_2), 7.45−7.75 (m, 9H, biphenyl).

Assay of AE Activity. The assay of AE activity consisted of three phases: (1) formation of the agonist-A_1AR-G protein ternary complex; (2) stabilization of that complex by the AE, and (3) dissociation of the complex by adding a combination of an A_1AR antagonist to compete with agonist at the orthosteric site and GTPγS to accelerate dissociation by displacing GDP from the G protein. The assay employed membranes from CHO-K1 cells stably expressing the hA_1AR. For agonist binding to equilibrium, the incubation mixture consisted of 10 mM HEPES, pH 7.2, containing 0.5 mM $MgCl_2$, 1 U/mL adenosine deaminase, 0.5 nM [^{125}I] N^6-(4-aminobenzyl)adenosine {[^{125}I]ABA}, and 10 μg of membrane protein ($B_{max} \cong 4$ pmol/mg protein) in a final volume of 100 μL. After 90 min at room temperature, the addition of 50 μL of a 0.3 mM solution of a candidate AE initiated stabilization of the ternary complex. Five minutes later, the addition of 50 μL of a solution of 400 μM 8-cyclopentyltheophylline and 200 μM GTPγS initiated the dissociation of the ternary complex. Ten minutes later, filtering through Whatman GF/C membranes, washing, drying, and counting ^{125}I-activity measured residual agonist. The percentage of specifically bound agonist remaining after 10 min of dissociation served as an index of AE activity

$$\text{AE activity} = 100 \times (B - B_o)/(B_{eq} - B_o)$$

where B = residual binding (cpm) bound at the end of 10 min of dissociation in the presence of an AE, B_o = residual binding (cpm) at the end of 10 min of dissociation in the absence of an AE, and B_{eq} = cpm bound at the end of 90 min of equilibration.

Assay of A_1AR Antagonist Activity. Assays of antagonism of equilibrium binding by allosteric enhancers used membranes from CHO-K cells expressing the hA_1AR. Assays, in triplicate, consisted of mixing 50 μL aliquots of membrane suspensions (15 μg of protein) in 10 mM HEPES, pH 7.4, containing 1 mM EDTA, 1 u/mL adenosine deaminase, and 4 nM [^{3}H]CPX with 50 μL of either 200 μM enhancer dissolved in HEPES buffer containing 10% methyl sulfoxide or, as controls, HEPES containing 10% DMSO. Additional aliquots of membrane suspension mixed with 50 μL of 200 μM NECA in HEPES−10% DMSO served for measurements of unspecific binding. Incubation for 3 h at room temperature established binding equilibrium. Filtration through Whatman GF/C membranes separated free and bound radioligand. The membranes were washed three times and dried, and ^{3}H activity was measured by liquid scintillation spectrometry. Inhibition was expressed as percentage of control specific binding. Table 2 reports the mean ± SEM of three separate assays.

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