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Allosteric Modulators of the Adenosine A_1 Receptor: Synthesis and Pharmacological Evaluation of 4-Substituted 2-Amino-3-benzoylthiophenes

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A series of 4-substituted 2-amino-3-benzoylthiophenes was screened using a functional assay of A_1AR-mediated phosphorylation of ERK1/2 in intact CHO cells to identify both potential agonistic effects as well the ability to allosterically modulate the activity of the orthosteric agonist, R-PIA. More detailed concentration−response experiments were subsequently performed on two compounds (**9a** and **9o**) utilizing both the ERK1/2 assay as well as a second assay of [^{35}S]GTPγS binding to activated G proteins.

Introduction

Allosteric modulation of the multimeric ion-channel GABA$_A$ receptor is a validated therapeutic approach that underlies the well-known mode of action of the benzodiazepines,[1] which act specifically at sites that are topographically distinct from the endogenous (orthosteric) agonist binding sites. Over the last two decades, the relatively unexplored concept of allosteric modulation of G protein−coupled receptors (GPCRs[a]) has also grown in interest to the point where it now represents one of the most exciting areas in modern drug discovery.[2] One GPCR system that is of particular interest with respect to allosteric modulation is the agonist, adenosine, and its cognate receptors.[3,4] Adenosine is an important tissue-protective agent that is released during ischemia, hypoxia, or inflammation to interact with multiple subtypes of GPCRs that can regulate a variety of effectors including adenylate cyclase and the ERK1/2 MAP kinases. Efforts to selectively target these receptors with modified adenosine analogs have resulted in therapeutics that are limited by side effects due to their ubiquitous nature and poor receptor subtype selectivity. The potential therapeutic applications of allosteric enhancers (AEs) at the adenosine A_1 receptor (A_1AR) are far more enticing than the classical agonist/orthosteric interactions alone, the idea being that the AE binds to a site that shows greater sequence divergence between adenosine receptor subtypes and also preferentially acts in the presence of elevated levels of endogenous adenosine. Thus, this approach can lead to greater selectivity in action as a consequence of both site- and receptor-specific modulation.

In 1990, Bruns et al.[5,6] reported that 2-amino-3-aroylthiophenes acted as AEs of the A_1AR by enhancing the binding of N^6-cyclopentyladenosine, although at higher concentrations of modulator, antagonistic effects were also observed. In that body of work, three compounds (**1**−**3**) were tested in substantial detail and were found to be effective AEs (Figure 1). PD 81,723 (**1**)[5] was the most potent, selective, and efficacious enhancer with the best ratio of enhancer/antagonistic activity. This research also highlighted the importance of the 2-amino and 3-carbonyl moieties as crucial substituents whose modification or removal greatly diminished enhancing activity. Following this work, a number of researchers in the field have probed the structure−activity relationships (SAR) of the 2-amino-3-carbonylthiophene core as the basis for the development of new and improved allosteric enhancers of the A_1AR. In 1999, van der Klein et al.[7] synthesized and evaluated LUF 5484 (**4**), an analog of PD 117,975 (**3**), and found it to be 2.4 times more potent than compound **1** while showing comparable antagonistic activity. An extension of this theme by Baraldi et al.[8] yielded the 3-naphthoyl derivatives **5a**−**e** with improved potency relative to **1** for increasing radiolabeled agonist binding to both human and rat A_1ARs.

Tranberg et al.[9] further investigated the structure−activity relationships of 2-amino-3-aroylthiophenes. In that study, the target compounds were screened using a radioligand dissociation kinetic assay that measured the ability of the candidate AEs to stabilize an orthosteric agonist−receptor−G-protein ternary complex. This research identified a number of 3-aroyl groups that support allosteric activity, and also suggested the presence of a second region in the allosteric binding site that interacts constructively with alkyl substituents in the 4- and 5-positions.

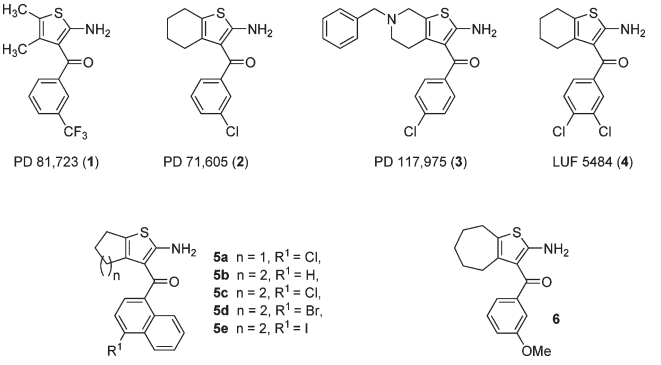

Figure 1

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[a]Abbreviations: AE, allosteric enhancer; AR, adenosine receptor; GPCR, G protein-coupled receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2.

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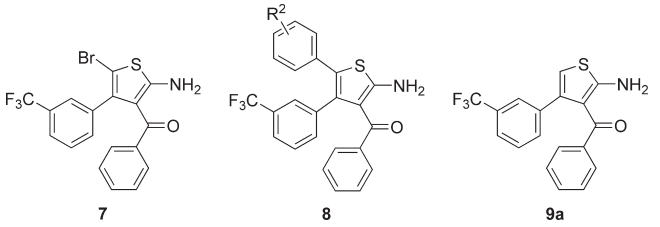


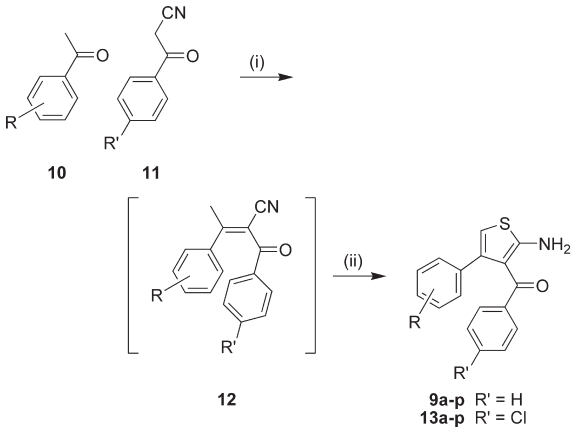
Figure 2

Lütjens et al.[10] synthesized a series of 4,5-disubstituted 2-amino-3-benzoylthiophenes and found that compound **7** (Figure 2) was effective and more potent than compound **1** in the kinetic binding assay described above. This was the first example of a 5-bromothiophene with AE activity equal to or greater than compound **1**. This finding prompted a broader study of the structure−activity relationships of the 5-position of compound **7**.[11] A series of 5-phenyl moieties with both electron donating and withdrawing activity were prepared and evaluated as A_1AR allosteric enhancers. The 5-phenyl analog **8** (R^2 = H) was the most potent of the series. However, the precursor with no substituent in the 5-position (compound **9a**) proved to be the most efficacious. The current study involved the synthesis of analogs of **9a**, with attention to the 3- and 4-positions, incorporating different substituents on the phenyl rings and the use of cell-based assays of A_1AR-mediated signaling in order to validate the functional allosteric properties of **9a** and its analogs as well as obtaining novel insights into their modes of action.

Results and Discussion

Chemistry. The targeted 3- and 4-substituted 2-aminothiophenes were prepared from the appropriately substituted acetophenone **10** and benzoylacetonitrile **11** via a two-step procedure that involved a Knoevenagel condensation followed by a Gewald reaction (Scheme 1). A one-pot Gewald procedure was not employed in this study as it is well documented that aryl ketones are unreactive in this procedure[12] and that the common method for preparing these thiophenes is via the two-step technique by initially forming the α,β-unsaturated nitriles **12** under Knoevenagel condensation conditions (β-alanine, AcOH, benzene, or toluene reflux) and then cyclizing to the thiophene with elemental sulfur and base.[13,14] This approach utilizes the classical azeotropic removal of water in the condensation step and in larger scale is quite versatile, whereas in our hands, this technique fails to produce adequate yields on a smaller scale. Therefore, the modified version of the condensation step developed by Lehnert,[15] which utilizes titanium(IV) chloride/pyridine system, provided low to moderate yields of α,β-unsaturated nitriles **12** that were subsequently cyclized to the thiophenes **9a**−**p** and **13a**−**p**. Attempts to drive the condensation step to completion by increasing the amount of titanium(IV) chloride resulted in lower yields and in some cases total consumption of acetophenones was observed without sign of intermediate **12**, as revealed by TLC and NMR. It was found that 1 equivalent of titanium(IV) chloride was sufficient to allow formation of **12**, and in the case of forming **9a**, utilizing one equivalent of titanium(IV) chloride compared to two equivalents[11] improved the yield by 7% after recrystallization from isopropanol. 2′-Substituted acetophenones were submitted for Knoevenagel condensation, and it was found that the 2′-fluoroacetophenone did condense, whereas

Scheme 1[a]



[a] (i) $TiCl_4$, pyridine, CH_2Cl_2; (ii) sulphur, Et_2NH, THF.

2′-chloroacetophenone produced only trace amounts (as determined by TLC and NMR) and 2′-bromoacetophenone did not provide any Knoevenagel product **12**. This was also the case for 2′-trifluoromethylacetophenone and other 2′-substituted acetophenones larger than chlorine. Thiophenes **9a**−**p** and **13a**−**p** were isolated and purified by silica gel chromatography and recrystallized from an appropriate solvent.

The yield of the Knoevenagel−Gewald sequence was modest at best (ranging from 3 to 40%), although adequate amounts of the desired products were obtained for pharmacological evaluation. Improved methodology was sought in order to facilitate the scale-up and further evaluation of the compounds of this type. We have recently found that yields can be improved by using phenacyl bromide or chloride rather than acetophenone and modified conditions in the Gewald step (NaSH was used instead of elemental sulfur and an organic base). In a representative example, compound **9b** was synthesized in this fashion and twice the yield (60%) of that previously achieved from acetophenone was obtained.

Biological Activity. To assess the biological activity of the putative AEs, compounds were initially screened using an AlphaScreen plate-based assay of A_1AR-mediated phosphorylation of ERK1/2 (pERK1/2) in intact CHO cells.[16] For each compound, two concentrations (3 and 10 μM) were tested alone and against an EC_{50} concentration of the orthosteric agonist, R-PIA. Under these conditions, an increase in functional response represents either an allosteric enhancement of R-PIA function or intrinsic agonism of the test compound (or both). An EC_{50} concentration of R-PIA was chosen to allow for the detection of either positive of negative allosteric modulators with limited degrees of cooperativity.[18] It should be noted that the use of only two concentrations of orthosteric agonist in these initial screens may fail to detect compounds with more complex profiles of A_1 receptor modulation (e.g., mixed allosteric/orthosteric modes), but the emphasis of the current study was to follow-up only on compounds that were unambiguously acting as potentiators over the concentration ranges utilized. As indicated in Table 1 and Figure 3B, a range of compounds were identified that demonstrated a substantial functional potentiation of the ability of R-PIA to promote A_1AR-mediated ERK1/2 phosphorylation. In addition, some compounds clearly displayed agonistic activity in their own right (Figure 3A). The most robust degree of potentiation exhibited by a number of the compounds, including **9a**, resulted in approximately 90% or more of the maximum attainable

Table 1. Effect of Test Compounds on A_1AR-Mediated Stimulation of ERK1/2 Phosphorylation in Intact CHO FlpIn Cells in the Presence of an EC_{50} Concentration of R-PIA

	structure		activity[a]	
no.	R′	R	3 μM	10 μM
9a	H	3-CF_3Ph	83 ± 3	59 ± 1
9b	H	Ph	49 ± 1	48 ± 2
9c	H	2-FPh	55 ± 1	68 ± 3
9d	H	3-FPh	51 ± 1	52 ± 2
9e	H	4-FPh	51 ± 1	60 ± 5
9f	H	3-ClPh	45 ± 1	52 ± 3
9g	H	4-ClPh	46 ± 1	51 ± 5
9h	H	3-BrPh	67 ± 9	76 ± 15
9i	H	4-BrPh	46 ± 1	49 ± 2
9j	H	4-IPh	51 ± 4	50 ± 1
9k	H	3-NO_2Ph	47 ± 2	50 ± 1
9l	H	4-NO_2Ph	50 ± 1	47 ± 2
9m	H	3-OCH_3Ph	41 ± 10	34 ± 17
9n	H	4-OCH_3Ph	52 ± 4	52 ± 3
9o	H	3,5-CF_3Ph	83 ± 7	93 ± 8
9p	H	2-Naphth	55 ± 1	73 ± 2
13a	Cl	3-CF_3Ph	64 ± 8	73 ± 8
13b	Cl	Ph	51 ± 3	64 ± 11
13c	Cl	2-FPh	70 ± 3	87 ± 7
13d	Cl	3-FPh	76 ± 6	90 ± 10
13e	Cl	4-FPh	62 ± 2	61 ± 10
13f	Cl	3-ClPh	58 ± 1	75 ± 18
13g	Cl	4-ClPh	50 ± 1	50 ± 1
13h	Cl	3-BrPh	62 ± 9	69 ± 2
13i	Cl	4-BrPh	45 ± 1	46 ± 4
13j	Cl	4-IPh	54 ± 8	61 ± 8
13k	Cl	3-NO_2Ph	53 ± 1	42 ± 7
13l	Cl	4-NO_2Ph	47 ± 5	52 ± 4
13m	Cl	3-OCH_3Ph	58 ± 3	60 ± 6
13n	Cl	4-OCH_3Ph	51 ± 3	55 ± 3
13o	Cl	3,5-CF_3Ph	69 ± 9	74 ± 7
13p	Cl	2-Naphth	54 ± 4	67 ± 24

[a] Effect of two different concentrations (3 and 10 μM) of substituted (2-amino-4-phenylthiophen-3-yl)(phenyl)methanones on A_1AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells, in the presence of an EC_{50} concentration of R-PIA (determined on the same day as each assay). Data represent the mean ± standard deviation of two experiments conducted in triplicate.

R-PIA response by a concentration of agonist that normally yields 50% of the maximum response (Table 1, Figure 3). In general, the series that possessed a 4-chlorobenzoyl group in the 3-position of the thiophene (compounds **13a**−**p**) showed slightly higher activity than the corresponding 4-benzoyl series (**9a**−**p**). However, there were exceptions to this trend. In both series, 3-trifluoromethylphenyl (**9a**, **13a**) and 3,5-ditrifluoromethylphenyl (**9o**, **13o**) substitution of the 4-position supported robust activity. In the 4-benzoyl series, 2-fluorophenyl, 4-fluorophenyl, 3-bromophenyl, and 2-naphthyl substitution (compounds **9c**, **9e**, **9h**, and **9p**, respectively) all augmented the R-PIA response from 50% (in the absence of compound) to ≥60% of the maximum attainable R-PIA response. A wider range of analogs achieved this level of activity in the 4-chlorobenzoyl series, namely compounds **13a**−**13f**, **13h**, **13j**, **13m**, **13o**, and **13p**. In addition to those substituents described above, phenyl, 3-fluorophenyl, 3-chlorophenyl, 4-iodophenyl, and 3-methoxyphenyl also augmented the R-PIA response to ≥60% of the maximum attainable R-PIA response. A similar trend for activity was also seen for basal responses to the compounds.

To further validate the results of the initial functional screen, more detailed experiments were performed using reference compound **9a** and the novel compound **9o**, as both compounds exhibited strong functional potentiation in the initial screens. As shown in Figure 4, increasing concentrations of either compound caused a significant potentiation of the potency of R-PIA for mediating either ERK1/2 phosphorylation (Figure 4a) or [^{35}S]GTPγS binding to activated Gα protein subunits (Figure 4b). In agreement with the results of the initial ERK1/2-based screens, a substantial degree of R-PIA-independent receptor activation was also noted in both assays in response to the modulators, confirming that the AEs also behaved as allosteric (partial) agonists in their own right; similar agonistic effects of A_1AR AEs have been noted in the past[17] and are consistent with the notion that part of the mechanism of action of these compounds is to stabilize an active receptor−G protein ternary complex. Because complete concentration−response profiles were determined for **9a** and **9o** using the two functional assays, we were also able to apply a novel operational model of allosterism[18,19] (see Supporting Information) to the data to derive quantitative estimates of the affinity of each modulator for the allosteric site on the unoccupied A_1AR (expressed as a pK_B; negative logarithm of the dissociation constant) as well as a measure of the cooperativity (Log $\alpha\beta$) exerted on the actions of R-PIA by the modulators. The latter parameter reflects the magnitude of the allosteric effect of the modulator on R-PIA affinity (α) and R-PIA signaling efficacy (β); under the present experimental conditions, individual estimates of these parameters could not be obtained, but the composite cooperativity factor, $\alpha\beta$, was determined as a measure of the overall allosteric effect. For the ERK1/2 experiments, the pK_B of **9a** was estimated as 6.37 ± 0.16 and Log $\alpha\beta$ as 0.38 ± 0.07 (i.e., $\alpha\beta$ = 2.4-fold positive cooperativity), whereas for the [^{35}S]GTPγS assay, the values were pK_B = 5.96 ± 0.17 and Log $\alpha\beta$ = 0.84 ± 0.07 (i.e., $\alpha\beta$ = 6.9-fold positive cooperativity). For compound **9o**, the parameters were pK_B = 5.22 ± 0.07 and Log $\alpha\beta$ as 1.06 ± 0.08 (i.e., $\alpha\beta$ = 11.5-fold positive cooperativity) for the stimulation of ERK1/2 phosphorylation and pK_B = 5.82 ± 0.27 and Log $\alpha\beta$ = 1.42 ± 0.16 (i.e., $\alpha\beta$ = 26.3-fold positive cooperativity) for the [^{35}S]GTPγS assay. There was no significant difference between the two pK_B values ($p > 0.05$, unpaired t test) for either AE between signaling assays as would be expected because the parameter reflects the affinity of the modulator for the allosteric site on the unoccupied A_1AR and should be independent of the assay used to determine the value. In addition, there was no significant difference ($p > 0.05$, unpaired t test) between the estimates of positive cooperativity exerted on the signaling of R-PIA by **9o** between the two different functional assays, indicating that the degree of allosteric potentiation is similar irrespective of which signaling pathway is investigated. However, there was a significant difference ($p < 0.05$, unpaired t test) between the estimates of positive cooperativity exerted on the signaling of R-PIA by **9a** between the two different functional assays. This suggests that **9a** may display functional selectivity with respect to the degree of allosteric potentiation it can exert on a given signaling pathway. Such "stimulus trafficking" by orthosteric compounds is a phenomenon that has gained significant recognition in the GPCR field in recent years,[20] and it is quite possible that allosteric modulators can also engender this property by promoting unique receptor conformations that differentially affect one signaling pathway relative to another.[19]

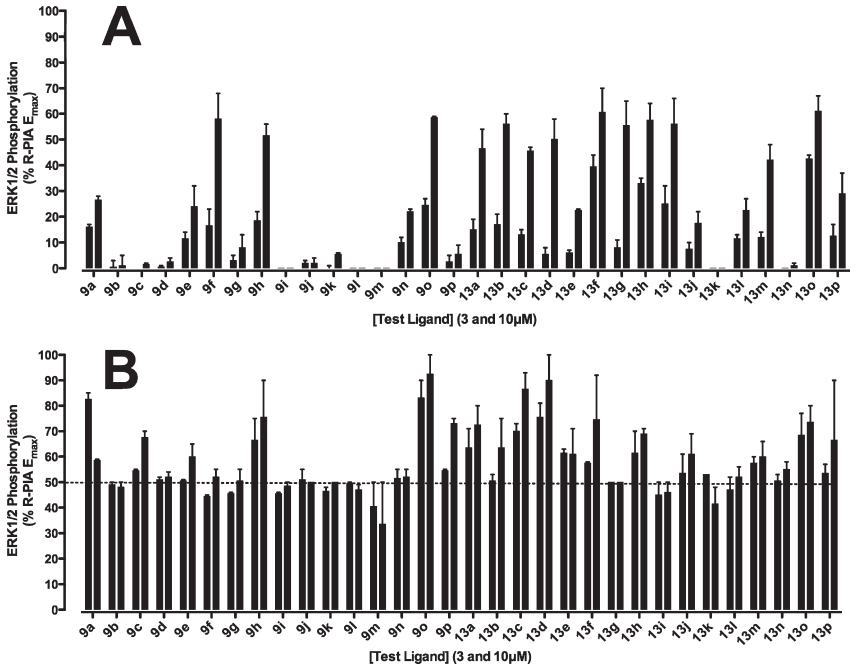


Figure 3. Effect of two different concentrations (3 μM, left bar; 10 μM, right bar) of novel 4-substituted 2-aminothiophenes on A_1AR-mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells, in the absence (A) or presence (B) of an EC_{50} concentration of R-PIA (determined on the same day as each assay). Data represent the mean ± standard deviation of two experiments conducted in triplicate.

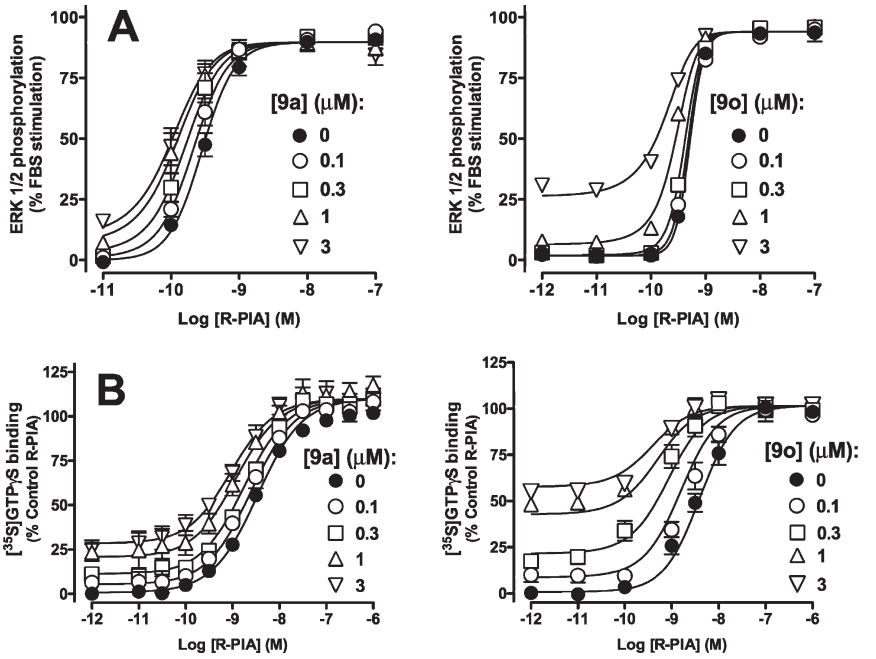


Figure 4. Effect of compounds **9a** or **9o** on (A) R-PIA mediated stimulation of ERK1/2 phosphorylation in intact CHO FlpIn cells and (B) R-PIA mediated binding of [^{35}S]GTPγS (100 pM) to activated G proteins in membranes from CHO FlpIn cells stably expressing the human A_1AR. Data points represent the mean ± SEM of three experiments conducted in triplicate. Curves drawn through the points represent the best global fit of an operational model of allosterism to the data sets.

Conclusion

In summary, a series of 4-substituted 2-amino-3-benzoylthiophenes and 2-amino-3-(4-chlorobenzoyl)thiophenes were prepared using a two-step sequence involving a Knoevenagel condensation and Gewald reaction. An initial screen supplemented by more comprehensive functional assays identified and validated novel 4-substituted 2-aminothiophenes as potent functional AEs of A_1AR-mediated signaling. In addition to a potential for exhibiting greater selectivity of action by targeting an allosteric site on the A_1AR, some of these compounds may also display signaling pathway bias via allosterically engendering functional selectivity in the actions of orthosteric agonists at the same receptor.

Experimental Section

General Procedure for the Preparation of Compounds 9a−p and 13a−p (Method A). The appropriate benzoylacetonitrile **11** (3 mmol) and acetophenone **10** (3.3 mmol) were dissolved in dry dichloromethane (12 mL) in a two-neck flask in an N_2

atmosphere and cooled to 0 °C with an ice bath. To the cooled solution was added dropwise neat $TiCl_4$ (3.3 mmol, 362 μL). After approximately 10−30 min, dry pyridine (215 μL) was added dropwise and the ice bath was removed. After 1 h, a further aliquot of dry pyridine (644 μL) was added dropwise and left to stir at room temperature overnight. The mixture was diluted with 2 M HCl (30 mL) and the organic phase separated. The aqueous phase was extracted with CH_2Cl_2 (2 × 30 mL), and the combined organics were washed with water and then brine, dried over $MgSO_4$, filtered, and concentrated to a resin. The resin was taken up in THF (6 mL), and elemental sulfur (3.3 mmol, 106 mg) was added, followed by Et_2NH (610 μL), and stirred at room temperature for 18 h. The mixture was diluted with ethyl acetate and washed with water (×2) and then brine, dried over $MgSO_4$, filtered, and concentrated to a resin. The resin was chromatographed on silica gel eluting with 10−30% ethyl acetate, petroleum ether (40−60 °C), providing after concentration of the appropriate fractions a solid that was recrystallized from isopropyl alcohol. Compounds **9k**−**l** and **13k**−**l** were recrystallized from ethyl acetate, and compounds **9o** and **13o** were recrystallized from petroleum ether (40−60 °C). Analytical HPLC was conducted on a Waters 2690 instrument with 996-diode array detector (chromatograms show UV absorbance at 254 nm) on a Phenomenex C8 (5 μm, 150 × 4.6 mm^2) column. All compounds were of ≥98% purity.

Alternative Procedure for the Preparation of Compound 9b (Method B). Benzoylacetonitrile **11** (0.94 mmol) and phenacyl bromide (1.03 mmol) were dissolved in dry CH_2Cl_2 (4 mL) in a two-necked flask fitted with a rubber septum and nitrogen inlet. The mixture was cooled with an ice−water bath, and neat $TiCl_4$ (206 μL) was added dropwise. After 0.5 h, dry pyridine (67 μL) was added dropwise and the ice−water bath was removed. After stirring a further 1 h, another aliquot of dry pyridine (200 μL) was added dropwise and the reaction mixture left to stir overnight. The reaction mixture was partitioned between 2 M HCl (50 mL) and CH_2Cl_2 (20 mL). The phases were separated, and the aqueous layer was extracted with DCM (2 × 20 mL). The combined organic phases were washed with water and finally brine, dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give the *E*/*Z* mixture of olefins as an amber resin or oil. The crude mixtures were used directly in the next reaction without purification.

The *E*/*Z* mixture was dissolved in EtOH (5 mL) and cooled to −78 °C (MeOH/dry ice) and a solution of NaSH (2.0−2.2 mmol) in EtOH (8 mL) was added dropwise over 30−60 s. The reaction mixture was left to stir while the MeOH/dry ice-bath was kept in place, but no further additions of dry ice were made. The MeOH/dry ice-bath slowly warmed up to 10 °C (approximately 1 h), and a small amount of precipitate was observed. The mixture was diluted with CH_2Cl_2 and while stirring 2 M HCl (20 mL) was added slowly with gas evolution occurring (CAUTION! H_2S is generated, which is highly toxic!). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2. The aqueous layer was treated with house hold bleach to oxidize residual H_2S. The combined organics are dried ($MgSO_4$), filtered and concentrated under reduced pressure in a fume cupboard, to a solid. The solid is recrystallized with isopropyl alcohol, providing **9b** as a yellow powder (157 mg, 60% yield). This material was identical in all respects to **9b** as synthesized via method A above.

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Note Added after ASAP Publication. This paper was published June 10, 2009 with an error in Figure 1. The revised version was published on June 15, 2009.

Supporting Information Available: Full details of the product purity and characterization and the pharmacological evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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