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Molecular features crucial to the activity of pyrimidine benzamide-based thrombopoietin receptor agonists

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Abstract—The identification of small molecule modulators of biological processes mediated via protein–protein interactions has generally proved to be a challenging endeavor. In the case of the thrombopoietin receptor (TPOr), however, a number of small molecule types have been reported to display biological activity similar to that of the agonist protein TPO. Through a detailed analysis of structure–activity relationships, X-ray crystal structures, NMR coupling constants, nuclear Overhauser effects, and computational data, we have determined the agonism-inducing conformation of one series of small molecule TPOr agonists. The relationship of this agonism-inducing conformation to that of other series of TPO receptor agonists is discussed.

Over the past two decades, an understanding of protein–protein interactions (PPIs) that play key roles in biological processes has led to intense pursuit of small molecules that can modulate these interactions.[1,2] Often this pursuit has focused on modulating interactions between the cell surface receptors and the proteins with which these endogenously interact. Both antagonism and agonism of receptor response have been sought depending on the biological function of the protein of interest. For example, in the type I cytokine receptor family,[3] small molecule antagonists of the ligand–receptor interaction of interleukins-2,[4–8] -5,[9,10] and -6,[11] and tumor necrosis factor-α[12] have been described. Examples of agonists in this receptor family include compounds acting on the receptors of the hematopoietic growth factors, granulocyte-colony-stimulating factor,[13–15] erythropoietin,[16–18] and thrombopoietin.[19–25]

Despite the effort expended on these and other PPIs, few of the reported small molecule antagonists or agonists constitute more than singular examples and fewer still have progressed into development as potential drugs. Agonists of the TPOr are an exception in that a number of drug-like small molecule agonists have been described,[19–31] one of which has been advanced into clinical studies and whose registration is pending.[32–35] Thus, in contrast to the modulation of other PPIs, the prospect is high that a TPOr agonist-based therapy will be introduced. Herein, we analyze a series of pyrimidine benzamide-based agonists (represented by **1**, Fig. 1), relating the SAR to key structural features and to physicochemical properties and computational data. The structural features of this and other TPOr agonist series are compared and discussed in the context of their interaction with the TPOr.

During analysis of the data generated with pyrimidine benzamide-based agonists,[25] we made a number of observations, some of which appeared to be trends. First, we noted that as the size of substituents at C-2

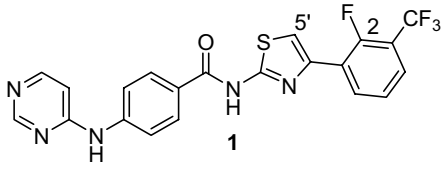

Figure 1. Example pyrimidine benzamide TPOr agonist.

Keywords: Thrombopoietin agonist; Nuclear Overhauser effects; X-ray crystal structure.
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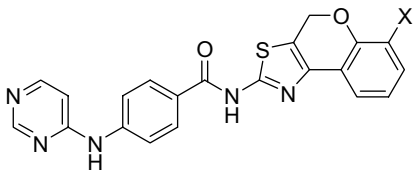
of the pendant phenyl ring (Table 1, R^2) increases, potency decreases (trend 1). Second, the 2,6-difluoro analog **10**, showing no agonism at the highest concentration examined, is substantially less active than the mono-fluoro analog **3** ('trend' 2). Third, the most potent compounds, whether in a mono-, di- or tri-substituted series, possess the largest lipophilic group at C-3 of the pendant phenyl ring (R^3) (trend 3). The latter trend was also noted in a series of tricyclic analogs (Table 2). Lastly, in comparing each 3- or 4-mono-substituted or 3,4-disubstituted analog with the corresponding compound also possessing a 2-fluoro group, we noted that the potency increases in all cases (trend 4).

The trends observed in analyzing the SAR of the pyrimidine benzamide series led us to formulate a hypothesis that co-planarity of the thiazole/phenyl ring portion of the molecules and the projection of a lipophilic group along a particular vector are critical to agonist activity. That co-planarity is important is supported by the first and second trends in which larger C-2 substituents or 2,6-disubstitution, both of which would be expected to induce a non-co-planar conformation, lead to dimin-

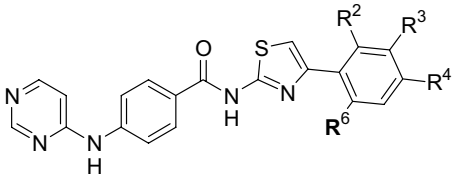
Table 2. Tricyclic 2-aminothiazole derived agonists[a]



Compound	X	EC_{50} (μM)
30	H	0.52
31	F	1.5
32	Cl	0.53
33	CF_3	0.14

[a] Ab initio calculations indicate a 10° dihedral angle between the thiazole and phenyl rings.

Table 1. Structure—TPOr agonist activity trends

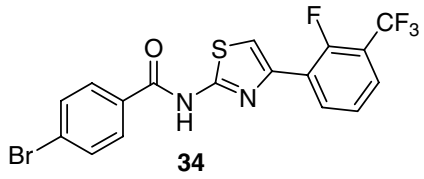


Compound	R^2	R^3	R^4	R^6	Trend comparison sets[a]				EC_{50} (μM)
					1st	2nd	3rd	4th	
2	H	H	H	H	A1	—	A1	—	3.4
3	F	H	H	H	A2	A	B1	—	6.8[b]
4	Cl	H	H	H	A3	—	C1	—	9.0
5	CF_3	H	H	H	A4	—	—	—	>10[b]
6	F	Cl	H	H	B1	—	B3	A2	0.61
7	Cl	Cl	H	H	B2	—	C2	—	1.3
8	F	H	Cl	H	C1	—	—	B2	0.23
9	Cl	H	Cl	H	C2	—	—	—	0.51
10	F	H	H	F	—	A	—	—	>10[b]
11	H	F	H	H	—	—	A2	C1	>10[b]
12	F	F	H	H	—	—	B2	C2	0.42
13	H	H	F	H	—	—	—	D1	2.6
14	F	H	F	H	—	—	—	D2	0.17
15	H	Cl	H	H	—	—	A3	A1	3.0
16	H	H	Cl	H	—	—	—	B1	0.57
17	H	Br	H	H	—	—	A4	E1	0.64
18	F	Br	H	H	—	—	B4	E2	0.10
19	H	CF_3	H	H	—	—	A5	F1	0.42
1	F	CF_3	H	H	—	—	B5	F2	0.060
20	H	H	CF_3	H	—	—	D1	G1	0.49
21	F	H	CF_3	H	—	—	E1	G2	0.26
22	H	OCF_3	H	H	—	—	A6	H1	0.28
23	F	OCF_3	H	H	—	—	B6	H2	0.032
24	H	F	F	H	—	—	F1	I1	0.40
25	F	F	F	H	—	—	G1	I2	0.084
26	H	CF_3	F	H	—	—	F2	J1	0.10
27	F	CF_3	F	H	—	—	G2	J2	0.033
28	H	F	CF_3	H	—	—	D2	K1	0.29
29	F	F	CF_3	H	—	—	E2	K2	0.087

[a] See Supplementary Materials for Table 1 sorted by the 3rd and 4th trends.
[b] Activity at 10 μM: **3**—58%, **5**—38%, **10**—0%, **11**—45%.

ished potency. That a lipophilic group at C-3 of the pendant phenyl group is important is supported by the third trend in which the most potent analog in each sub-series of compounds possesses the largest lipophilic group at C-3. That this lipophilic group is most effective in inducing agonism when it projects along a vector generally anti to the thiazole ring N is supported by the fourth trend in which each 2-F susbstituted analog is more potent than the corresponding analog in which the 2-F group is absent. In this case, we reasoned that, owing to a local dipole effect, the 2-F group induces the conformation in which the 2-F group, and thereby the C-3 substituent, is in an orientation anti to the thiazole ring N. Our hypothesis is further supported by the tricyclic analogs (Table 2), which are planar, have a lipophilic group projecting along the proposed vector, and the analog with the largest lipophilic group, **33**, is the most potent.

Data from a number of other sources supported our hypothesis. First, as is observed in the crystal structure of **34**, a bromobenzamide related to agonist **1**(Fig. 2),[36] the thiazole and its pendant substituted phenyl group are nearly co-planar (dihedral angle 5.7°) and oriented such that the 2-fluoro substituent is anti to the thiazole ring N atom. The dihedral angle between the amide and the thiazole is 6.7° and that between the amide carbonyl and the benzene ring to which it is directly bonded is 21.2°. Thus, in the solid state, and at least for this polymorph of **34**, the proposed agonism-inducing conformation is observed. The X-ray crystal structure of **35**, a TPOr agonist from the thiazolidine-thiazole series,[28,36] revealed similar features (Fig. 3); the dihedral angle between the aryl rings is 17.5° and that between the amide carbonyl and the directly-bonded benzene ring is 22°.



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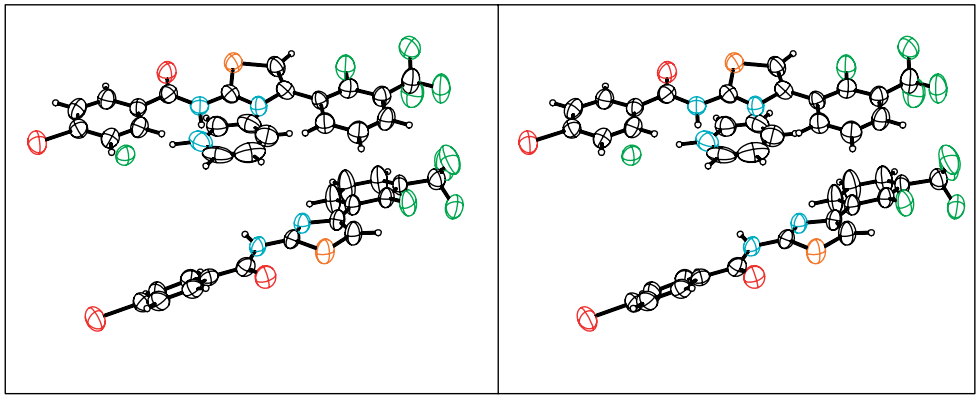
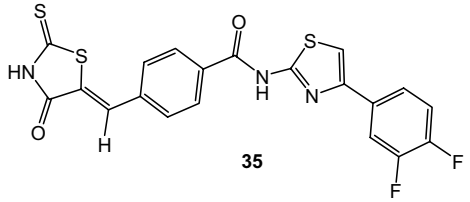


Figure 2. X-ray crystal structure of **34** (co-crystallized with pyridine hydrochloride).



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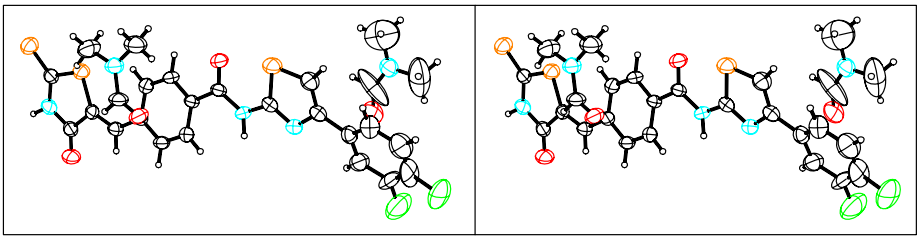


Figure 3. X-ray crystal structure of a thiazolidine-thiazole TPOr agonist **35** (co-crystallized with dimethylformamide).

Solution state data, in the form of long range fluorine–proton and fluorine–carbon couplings in the NMR spectra, also supported the hypothesis. During the course of our work, we noted that 4-(2-fluorophenyl)thiazole-containing compounds generally display a long-range coupling, $^{5}J_{F-H}$, between the thiazole C-5′ proton and the 2-fluoro group (Table 3, **36** and **38**–**41**). An exception to this was **37** which did not display a $^{5}J_{F-H}$ coupling. That aminothiazoles **36** and **38**–**41** display $^{5}J_{F-H}$ coupling constants, while **37** does not, indicates that in the case of the former set the 2-F and 5′-H are in close proximity to each other. $^{5}J_{F-H}$ and longer couplings have been observed in other systems and the magnitude of the coupling has been shown to be dependent on the proximity in space of the coupled atoms.[37–41] For example, 4-fluorophenanthrene displays a $^{5}J_{F-H}$ coupling of 2.6 Hz while 7-fluorobenzo[b]fluoranthrene displays no corresponding $^{5}J_{F-H}$ coupling (Fig. 4).[37,41] Since no $^{5}J_{H-F}$ coupling is observed in the 2,6-difluoro analog, **37**, we conclude that the C-5′ thiazole proton is not proximal to either fluorine atom. Thus, the thiazole and phenyl rings of **37** are not co-planar, which is not unexpected for an ortho, ortho-disubstitued biaryl system. A corollary to this conclusion is that an in-plane fluorine atom syn to the thiazole ring N is destabilizing.

Examination of the $^{4}J_{F-C}$ couplings leads to the same conclusions; systems expected to have co-planar biaryl rings, **36** and **38**–**41**, display significant couplings between the 2-fluoro group and C-5 of the thiazole, while **37** displays only a small coupling.[38,41,42]

The effects of conformation on $^{5}J_{F-H}$ and $^{4}J_{F-C}$ couplings were further illustrated in analogs containing thiazole C-5′ substituents, **42**–**43** and **45**. Owing to the ortho–ortho′ biaryl substitution pattern and the destabilization of an in-plane fluorine atom syn to the thiazole ring N, the former two of these are expected to have non-co-planar biaryl systems and in both cases none of the possible $^{4}J_{F-C}$ couplings were observed. Furthermore, although **42** displays a $^{5}J_{F-F}$ coupling of 43.6 Hz, its magnitude also suggests that the biaryl system is not co-planar; a 4,5-difluorophenanthrene displays a $^{5}J_{F-F}$ coupling of 170 Hz.[37,40] In contrast, **45**

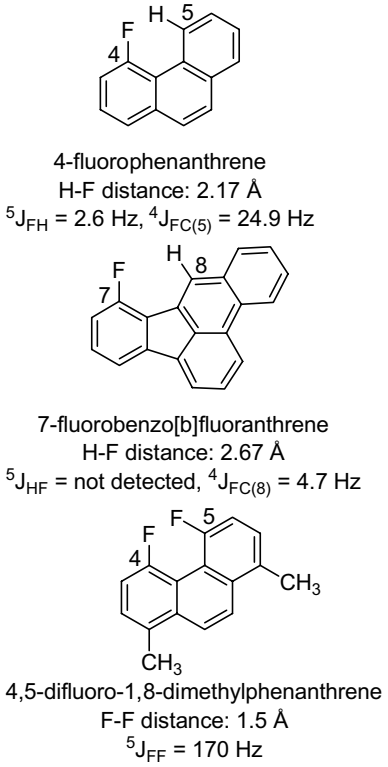
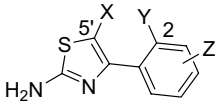


Figure 4. Examples of direct $^{5}J_{HF}$ and $^{4}J_{CF}$ couplings.[34,36,37]

Table 3. $^{5}J_{X-Y}$ and $^{4}J_{F-C}$ coupling constants of various 2-amino-4-phenylthiazoles[a] and BaF3 activity of corresponding pyrimidine benzamide or isonipecotic acid agonists (EC_{50} μM)



Thiazole	X	Y	Z	$^{5}J_{2Y-5'X}$ (Hz)	$^{4}J_{2F-5'C}$ (Hz)	$^{4}J_{C2(6)-5'F}$ (Hz)	EC_{50}	Agonist
36	H	F	H	1.6	11.3	—	6.8	**3**[b]
37	H	F	6-F	nd[c]	3.0	—	>10[d]	**10**[b]
38	H	F	3-OCF_3	1.7	11.6	—	0.032	**23**[b]
39	H	F	3-CF_3	2.6	14.7	—	0.060/0.057	**1**[b]/**47**[e]
40	H	F	4-CF_3	2.5	15.8	—	0.260	**21**[b]
41	H	F	5-CF_3	2.6	15.1	—	5.2	**48**[b]
42	F	F	3-CF_3	43.6	nd[c]	nd[c] (nd[c])	0.70	**49**[e]
43	CH_3	F	3-CF_3	—	nd[c]	—	0.54	**50**[b]
44	H	H	3-CF_3–4-F	nd[c]	—	—	0.10	**51**[b]
45	F	H	3-CF_3–4-F	~2	—	~5 (~8)	0.089	**52**[e]
46	CH_3	H	3-CF_3–4-F	—	—	—	0.18	**53**[b]

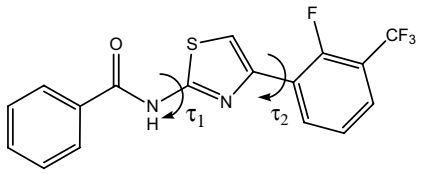
[a] $^{5}J_{2F-5'H}$ couplings were also observed in the agonists.
[b] Pyrimidine benzamide series.[25]
[c] nd, none detected.
[d] 0% agonist response at 10 μM.
[e] Isonipecotic acid series.[26,27,46]

displays $^{5}J_{F-H}$ and $^{4}J_{F-C}$ couplings indicating that conformations wherein the 5′-F group is in close proximity to either C-2 or C-6 are significantly populated. This is consistent with this mono-ortho-substituted biaryl system adopting a co-planar conformation.

Further corroboration of the conformational preferences was obtained through nuclear Overhauser effect (nOe) experiments examining energy transfer between proximal fluorine and hydrogen atoms. In the case of **36**, a nOe was observed between the C-2 fluoro group and both the adjacent C-3 proton and the C-5′ proton (ratio 5′:3–1.1). That the magnitude of these nOe effects are on the same order indicates that the two protons are nearly equidistant in space from the 2-F group, consistent with a co-planar biaryl system. In contrast, although a nOe between the C-2 fluoro group and the C-3 and C-5′ protons was observed for **37**, the effect on the C-5′ proton was much less than on the C-3 proton (ratio 5′:3–0.30) indicating that the 5′ proton was more distant from the 2-F group than the C-3 proton, consistent with a non-planar biaryl system. A nOe was also observed between the C-2 fluoro group and the C-5′ proton of **39**; however, in the absence of a proton at C-3 its relative magnitude could not be assessed.

Having established the conformational preferences of various 4-phenylthiazole systems, subsequent correlation with the agonist activity of key compounds further substantiated our hypothesis. Thus, **1** and **47**, which possess a co-planar biaryl system, are ~10-fold more potent than the corresponding analogs **50** and **49** which do not have co-planar biaryl systems (Table 3). That the loss of agonist potency in the latter two compounds in not due to the thiazole C-5′ substituent is indicated by the relative potency of the 4-F–3-CF_3 analogs, **52** and **53**, which, respectively, have, or are expected to have, co-planar biaryl systems. That a lipophilic group at C-3 of the pendant ring is optimal is supported by the activity trend of series **1**, **21**, and **48**, in which potency drops significantly as the lipophilic group is moved from C-3 to C-4 and then to C-5. That agonism is best stimulated when the lipophilic group has the steric bulk of at least a CF_3 group is supported by the potency trends of two sets of analogs in which the C-3 substituent is varied in size: H, F, Cl, CF_3 (Table 1: **3**, **12**, **6** and **1** and Table 2: **30**–**33**). In both sets, a significant difference in agonism is observed between the C-3 Cl and CF_3 substituted compounds.

Owing to our desire to avoid a potential toxicity issue with the 2-aminothiazole moiety in the pyrimidine benzamide series,[25] during the course of those studies we prepared a number of compounds in which the thiazole was replaced with other five-membered ring heterocycles. However, none of these provided as potent agonism as the thiazoles. In an effort to better understand this, we performed ab initio calculations on a series of benzamides corresponding to various relevant thiazoles and other heterocycles.[43] These calculations were also consistent with our hypothesis. For example, benzamide **54** (Fig. 5) was predicted to possess a single low-energy conformation with the 2-fluoro group anti to the thiazole ring N and co-planar aryl rings. Other thiazole/phenyl ring substituent patterns associated with potent activity were also predicted to possess the proposed agonism-inducing conformation. In contrast thiazole/phenyl ring substitution patterns not associated with potent activity were predicted to possess non-planar and/or multiple low-energy conformations. The latter also applied to benzamides of various thiadiazoles, oxazoles, pyrazoles, and isothiazoles (see Supplementary Materials).



54 ($\tau_1 = 1.5^{o}$ and $\tau_2 = 1.0^{o}$)

Figure 5. Calculated low-energy conformations of a thiazole benzamides ($\tau 1$ and $\tau 2 \approx 0°$ as shown).

Having established the agonism-inducing conformation of compounds from the pyrimidine benzamide series, comparison with other reported TPO agonists, for example, **35**, eltrombopag, and NIP-004, revealed a number of common elements. In addition to all being relatively flat molecules containing a terminal lipophilic-substituted benzene ring, each contains a Lewis basic group located approximately 15–17 Å away from the terminal benzene ring (Fig. 6). This Lewis basic group is likely key to the observation that these TPOr agonists are effective against the human receptor but not against the receptors of standard laboratory animals.[24] In humans, residue 499, which is located in the transmembrane domain, is histidine while in other species the corresponding residue is a hydrophobic residue, leucine or phenylalanine. Thus, a reasonable postulate is that the low molecular weight agonists interact with the receptor within the membrane and that hydrogen bonding, or salt bridge formation, between the histidine moiety and the Lewis basic group of the agonist is critical to induction of activity. Indeed, NMR experiments have revealed that an interaction between His-499 and the Lewis basic group of SB-394725 can be detected.[44] Owing to the low dielectric constant within the membrane,[45] the energetic magnitude of the interaction between the histidine and the Lewis basic group is likely to be substantial. Furthermore, owing to the low dielectric, non-polar environment, intramolecular hydrogen bonding, and local dipole effects will play a significant role in defining the geometry of a membrane-embedded molecule. Thus, the agonists as depicted (Fig. 5) may be highly rigidified by such interactions and thereby have the capacity to bind to and stabilize the higher energy, active conformation of the receptor's transmembrane domain. That a specifically substituted aryl ring ~15–17 Å distant from the Lewis basic group is also apparently required suggests that this portion is interacting with a particular region of the receptor transmembrane domain. Thus, in contrast to other attempts to modulate PPIs, wherein disruption or promotion of multiple, rel-

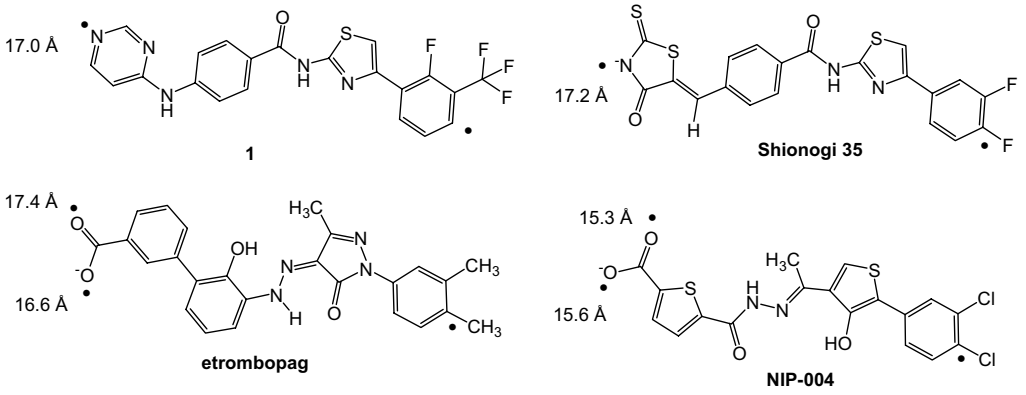


Figure 6. Comparison of TPOr agonists from four series. Distances from the Lewis basic groups to the C-4 carbon of the distal benzene ring are noted. Molecules are drawn in a two-dimensional format emphasizing potential hydrogen bonds and dipole interactions and do not represent computationally minimized structures.

atively low affinity interactions occurring across a large contact area has proven to be a challenge, the TPOr provides an example wherein a small molecule, through binding to a specific, localized region, modulates a receptor's function by stabilization of the active conformation. A more detailed understanding of the molecular nature of the interaction between such agonists and the TPOr is the subject of continuing studies that may reveal the prospects for identifying small molecule agonists or antagonists that interact in a similar fashion with other proteins.

Supplementary data

(1) NMR/MS data for **30**–**33** and NMR data for **36**–**45**, (2) nuclear Overhauser experiments for **36**, **37** and **39**, (3) Experimental Methods, (4) Discussion and table of ab initio calculations, (5) Alternate sorting of Table 1 columns. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.052.

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36. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC673644: **34**; CCDC673643: **35**. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].
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