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Synthetic Inhibitors of Extended Helix–Protein Interactions Based on a Biphenyl 4,4′-Dicarboxamide Scaffold

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The development of molecules that mimic the characteristics of α-helices has increased over the past few years because of their abundance in protein secondary structures and their key role in mediating protein–protein interactions.[1] Moreover, the applicability of α-helix mimetics as potential therapeutics in disease areas such as cancer, antimicrobials, and AIDS makes them attractive targets for molecular design.[2–6] The majority of the α-helix mimetic-based protein inhibitors reported to date can be subdivided into two categories. The first includes molecules that adopt helical structures similar to natural helices and most commonly include peptidomimetics such as β-peptides and peptoids.[7–10] The second ignores the helical structure of natural helices and instead uses scaffolds to project functionality in a similar spatial arrangement to the natural conformation.[1,2,11–14] In both cases, it is the strategic functionalization of the scaffolds that enables the development of inhibitors that effectively recognize biomacromolecules.

We have previously reported the design and synthesis of a low-molecular-weight mimetic based on an extended 3,2′,2″-trisubstituted terphenyl scaffold, **1**, that can project functionality in a similar orientation and distance as the key *i*, *i*+4, and *i*+7 residues on one face of an α-helix (Figure 1 A) and has been shown to serve as an inhibitor of the Bcl-x_L/Bak interaction.[15] A refinement of the design led to terephthalamide scaffold **2**, which has a simpler synthesis and improved solubility (Figure 1 B). In this new scaffold, the flanking rings of the terphenyl are replaced by two carboxamide groups and this introduces an intramolecular hydrogen bond between the amide –N–H and the alkoxy oxygen atom that influences the position of the amino acid side chain R^1. A straightforward synthesis incorporating O-alkylation and standard amide coupling reactions was used to prepare derivatives of **2**.[13] These terephthalamide inhibitors, like the terphenyls, were tested in a fluorescence polarization (FP) assay, and demonstrated nanomolar inhibition of the Bcl-x_L/Bak protein–helix interaction.[13]

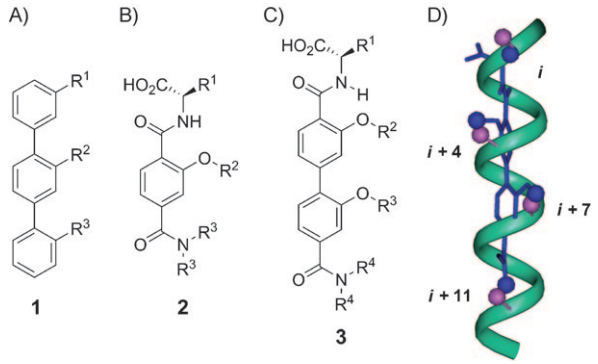

Figure 1. Structures of A) an ideal α-helix, terphenyl scaffold **1**, B) terephthalamide scaffold **2**, C) biphenyl scaffold **3**, and D) overlay of a poly-alanine α-helix (green, methyl groups highlighted in pink) and biphenyl **3 a** (blue, where $R^1 = R^2 = R^3 = R^4 = CH_3$) with RMSD = 1.368 Å.

Both the terphenyl and terephthalamide inhibitors were designed to mimic three residues of an α-helix. However, naturally occurring protein–helix and/or protein–protein interactions often occur through the contact of more than three residues, typically along one face of the helix. For this reason, molecules that can simultaneously mimic the *i*, *i*+3, *i*+4, and *i*+7 residues on two turns or the *i*, *i*+4, *i*+7, and *i*+11 residues on three turns would likely achieve a higher degree of specificity for their respective targets. We have recently reported strategies for four-residue mimetics[16] of α-helices as well as mimetics of extended α-helices.[17] Two scaffolds have been developed, the first of which was based upon the original terphenyl scaffold with an indane ring in place of the central phenyl ring.[16] Computational modeling shows good structural overlap between the side chains of the terphenyl indane and the *i*, *i*+3, *i*+4, and *i*+7 residues of an α-helix.[16] Herein, we report the design, synthesis, and in vitro application of a second scaffold, **3**, based on a biphenyl 4,4′-dicarboxamide structure designed to mimic the *i*, *i*+4, *i*+7, and *i*+11 residues of an α-helix (Figure 1 C). This scaffold combines the hydrophobic core of the oligophenyl series and the synthetically accessible carboxamide groups of the terephthalamides. An energy-minimized structure of biphenyl **3 a** (in which $R^1 = R^2 = R^3 = R^4 = Me$) shows good overlap with the *i*, *i*+4, *i*+7, and *i*+11 residue side chains of an α-helix with an RMSD value of 1.368 Å (Figure 1 D).

As shown in the representative synthesis (of **31**) in Scheme 1, biphenyls with varied side chains can be prepared by a Suzuki coupling of two functionalized aromatic amide subunits, **9** and **15**. The lower aromatic amide (**9**) is prepared from commercially available 4-amino-3-hydroxybenzoic acid (**4**). Methyl ester formation and subsequent O-alkylation afforded the 2-isopropoxy group in **6**. A Sandmeyer reaction was used to convert this aryl amine into the aryl iodide **7**. Hydroly-

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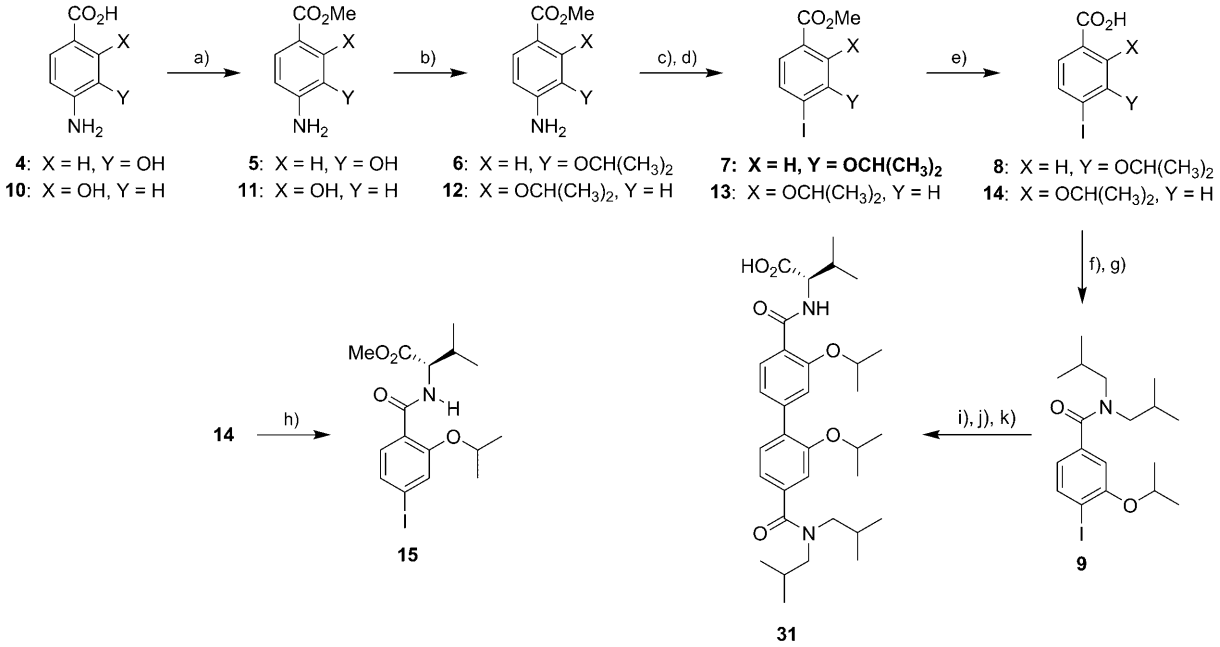


Scheme 1. Synthesis of **31**. a) H_2SO_4, MeOH, reflux; b) 2-iodopropane, Cs_2CO_3, acetone, reflux; c) $NaNO_2$, H_2SO_4, MeOH, H_2O, 0 °C; d) KI, Cu (bronze), reflux; e) LiOH, H_2O/THF; f) $(COCl)_2$, DMF, CH_2Cl_2, 0 °C; g) $(iBu)_2NH$, triethylamine, CH_2Cl_2, 0 °C; h) EDC, HOBt, L-valine methyl ester HCl, CH_2Cl_2, 0 °C; i) $Pd(OAc)_2$, KOAc, bis(pinacolato)diboron, DMF, reflux; j) **15**, Cs_2CO_3, $Pd(PPh_3)_4$, DMF, reflux; k) LiOH, H_2O/THF.

sis of this methyl ester, followed by acid chloride formation and coupling with diisobutylamine afforded **9**. The upper aromatic amide, intermediate **15**, was synthesized by using the same procedure as that of **4**–**8**, but starting from commercially available 4-amino-2-hydroxybenzoic acid (**10**); however, a standard peptide coupling reaction with the appropriate amino acid was used to prepare the amide derivative **15**. This intermediate was then used in a one-pot Suzuki coupling reaction[18] in which intermediate **9** was first converted into its boronate ester, followed by addition of **15** in the presence of $Pd(PPh_3)_4$. Finally hydrolysis gave biphenyl **31**.

An X-ray structure of **31** shows that an intramolecular hydrogen bond with an interatomic distance of 1.96 Å and an –N–H···O angle of 140.9° exists between the top amide –N–H and the alkoxy oxygen atom (Figure 2). This intramolecular hydrogen bond influences the position of the amino acid side-chain R^1 group. In addition, like the terphenyl, the biphenyl scaffold adopts a staggered conformation that projects the alkoxy R^2 and R^3 groups in a nonplanar arrangement similar to the spatial arrangement of naturally occurring α-helices. In the solid state, the overall conformation reinforces the staggered projection of side chains from the core, albeit with expected rotational flexibility in solution.

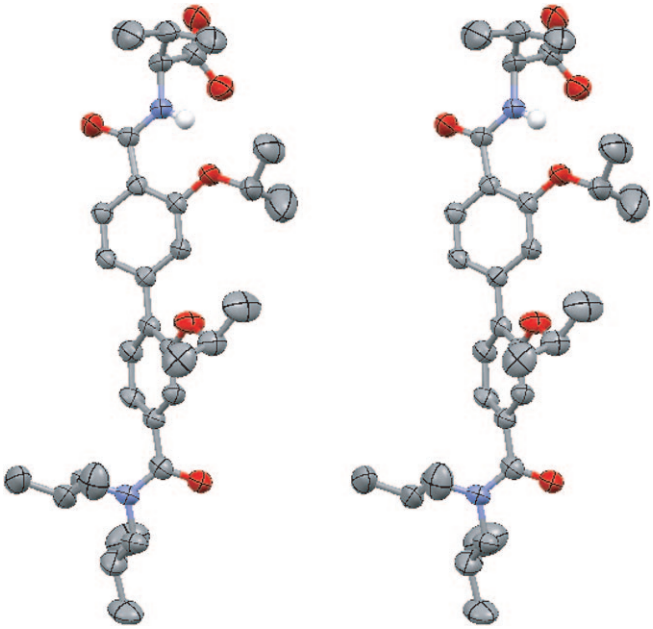


Figure 2. X-ray structure at the 70% level of **31** in stereoview.

In order to test if the biphenyl scaffold could effectively mimic an α-helix, we targeted the Bcl-x_L/Bak protein–helix interface.[15,19] Bcl-x_L and Bak are members of the Bcl-2 family of proteins, which are key regulators of the apoptotic pathway. Apoptosis is a fundamental process that is required for normal tissue development and is essential in maintaining cell homeostasis in multicellular organisms.[20] It is also one of the primary means for the destruction of cells that might be damaged and are harmful to the organism. Over the past decade, the apoptotic pathway has generated considerable interest since the discovery that a lack of apoptotic cellular regulation can lead to a wide range of illnesses, including cancer.[21–23] To date, many apoptosis-inducing therapeutic strategies have been reported[24] including the development of non-peptidic small molecules that disrupt protein–protein interactions in the apoptotic pathway.[25–27]

Elucidation by NMR spectroscopy of the binding interaction of the Bcl-x_L/Bak complex has provided a framework from which we can model and build a mimetic of the BH3 domain of Bak.[28] Fesik and co-workers found that the Bak peptide, an amphipathic α-helix, binds into a hydrophobic pocket, formed by the BH1, BH2, and BH3 regions of Bcl-x_L, by projecting the hydrophobic side chains Val74, Leu78, Ile81, and Ile85 into the cleft.[28] Furthermore, alanine scanning experiments showed a decrease in the binding affinity when residues corresponding to the *i*, *i*+4, *i*+7, and *i*+11 positions of the BH3 α-helix were substituted with alanine; thus indicating important functional contributions at these positions.

To test the effectiveness of our helix mimetics, we first employed a Bcl-x_L/Bak FP assay in which the α-helical BH3 domain of Bak was fluorescently labeled (Flu-Bak, Fl-GQVGRQ-LAIIGDDINR-$CONH_2$) and allowed to bind to Bcl-x_L. Displacement of this probe through competitive binding of α-helix mimetics into the hydrophobic pocket of Bcl-x_L leads to a subsequent decrease in fluorescence polarization. A plot of inhibitor concentration versus polarization values allows for the direct calculation of inhibition constants by using the K_d obtained from a direct titration of Flu-Bak with Bcl-x_L. The K_d of this one-site saturation experiment was found to be 15 nM, which is in accordance with the K_d values previously reported in the literature.[15]

The results of the FP competition assay of the biphenyls as antagonists of the Bcl-x_L/Bak complex are shown in Table 1, and representative titration curves are shown in Figure 3. In this first series of biphenyls, R^2 and R^3 were held constant as isopropoxy groups, based on the structure of the best terephthalamide inhibitor,[13] while the R^1 and R^4 groups were varied. Biphenyl derivatives with large hydrophobic *N*,*N*-alkyl substituents on the lower carboxamide (R^4) were found to be favored, while the dimethyl and diethyl amides (**17**–**24**) showed no inhibition, with K_i values weaker than 500 μM. In contrast, when the upper carboxamide (R^1) was varied, both small and large groups were tolerated with the best inhibition observed where R^1 was a methyl (**25**) or benzyl (**37**) group, with K_i values of 8.2 and 13 μM, respectively. In addition, when R^4 was an alkyl substituent, and R^1 was an isopropyl group, there was generally no observable or only weak inhibition (**18, 22, 26, 31**).

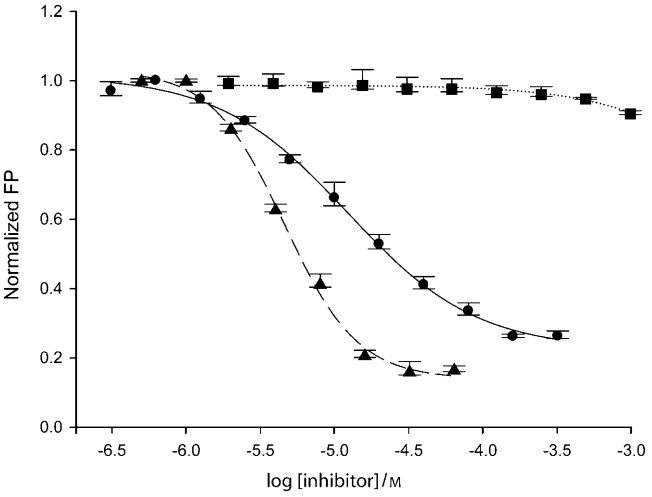


Figure 3. Titration curves for **50** (▲), **19** (■), and unlabeled Bak (●).

In order to probe the importance of the *R* or *S* configuration of the R^1 group, the D-amino acid derivatives of **27** and **37** were synthesized. This stereogenic center did not affect the affinity of the biphenyls significantly, as seen by the comparison of **27** and **28**, as well as that of **37** and **38**. Two additional biphenyl derivatives of lead compounds **25** and **37** lacking the bottom carboxamide group were prepared (**39** and **40**). These showed that the fourth functional group does aid in binding.

In a second series of biphenyl compounds, the R^2 and R^3 substituents were substituted with larger hydrophobic groups in order to increase the favorable hydrophobic contacts of the biphenyl with the surface of Bcl-x_L. These aryl-substituted biphenyls were synthesized in a similar manner to the bis-isopropoxy derivatives, with two modifications. First, the R^2 and R^3 groups were introduced by nucleophilic aromatic substitutions by using phenol and 1- and 2-naphthol with the corresponding aromatic fluorides (see the Supporting Information). Second, the Suzuki coupling reaction was performed as a two-step procedure instead of a one-pot reaction. In this case, the boronic acid of the bottom subunit was synthesized and purified prior to coupling to its corresponding partner. The Suzuki coupling reaction was then carried out with $Pd(PPh_3)_4$ and $NaHCO_3$ in a mixture of DME and water (15:1). Under these conditions, the biphenyl product is partially hydrolyzed. After acidic extraction of the reaction mixture, the crude product was treated with LiOH in THF/water (2:1) in order to convert the ester to the final product.

Overall, this second series of biphenyl derivatives showed better activity than their bis-isopropoxy counterparts (Table 2). These results indicate that an *iso*butyl group at the R^1 position is preferred over the smaller methyl substituent, as seen when comparing **42**, **44** and **46** with **41**, **43** and **45**, respective-

Table 1. Competition fluorescence polarization assay results for biphenyls **17**–**40**. R group naming follows Figure 1 C scaffold designation, with R^2 and R^3 held constant as isopropoxy functional groups.

#	R^1	R^4	K_i [μM]	#	R^1	R^4	K_i [μM]
17	Me	Me	>500	**18**	*i*Pr	Me	>500
19	*i*Bu	Me	>500	**20**	Bn	Me	>500
21	Me	Et	>500	**22**	*i*Pr	Et	>500
23	*i*Bu	Et	>500	**24**	Bn	Et	>500
25	Me	*i*Pr	8.2±0.62	**26**	*i*Pr	*i*Pr	140±54
27	(*S*)-*i*Bu	*i*Pr	15±6.5	**28**	(*R*)-*i*Bu	*i*Pr	30±6.5
29	Bn	*i*Pr	220±60	**30**	Me	*i*Bu	250±81
31	*i*Pr	*i*Bu	>500	**32**	*i*Bu	*i*Bu	120±40
33	Bn	*i*Bu	36±8.0	**34**	Me	Ph	58±7.6
35	*i*Pr	Ph	38±6.4	**36**	*i*Bu	Ph	28±6.9
37	(*S*)-Bn	Ph	13±3.5	**38**	(*R*)-Bn	Ph	39±8.0
39	Me	no amide[a]	420±82	**40**	Bn	no amide[a]	50±12

[a] See the Supporting Information for the chemical structures of **39** and **40** (Figure S6).

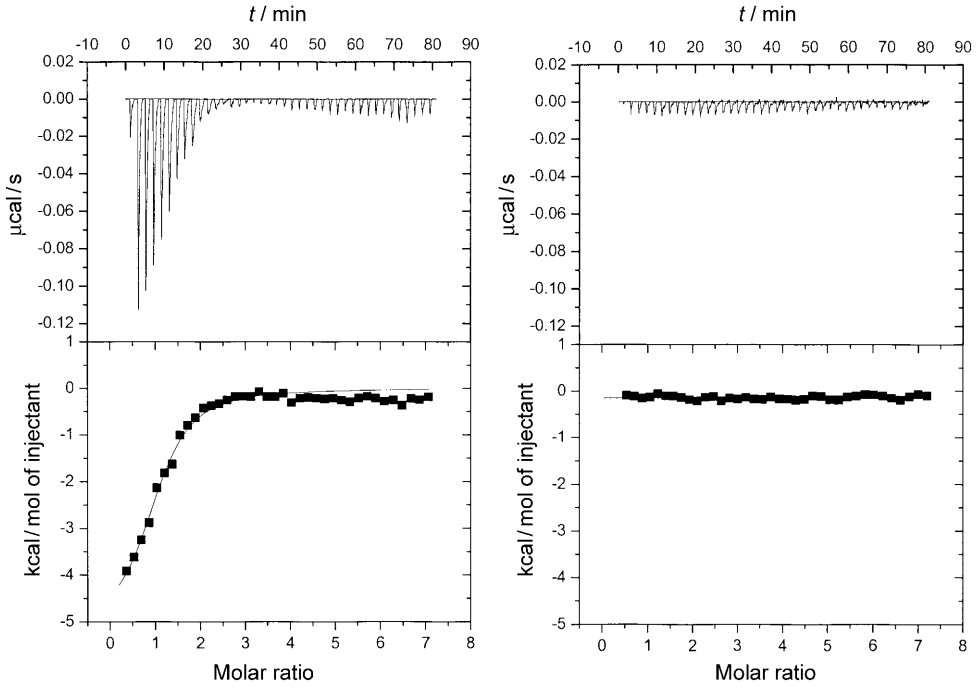
Table 2. Competition fluorescence polarization assay results for biphenyls **41**–**51**. R group naming follows Figure 1C scaffold designation.

#	R^1	R^2	R^3	R^4	K_i [μM]
41	Me	*i*Pr	Ph	*i*Pr	180 ± 55
42	*i*Bu	*i*Pr	Ph	*i*Pr	36 ± 6.3
43	Me	2-naphthyl	*i*Pr	*i*Pr	37 ± 7.0
44	*i*Bu	2-naphthyl	*i*Pr	*i*Pr	17 ± 6.1
45	Me	*i*Pr	2-naphthyl	*i*Pr	29 ± 6.2
46	*i*Bu	*i*Pr	2-naphthyl	*i*Pr	2.1 ± 0.57
47	Me	2-naphthyl	Ph	*i*Pr	8.6 ± 0.67
48	*i*Bu	2-naphthyl	Ph	*i*Pr	9.6 ± 0.57
49	Bn	*i*Pr	2-naphthyl	*i*Pr	> 500
50	*i*Bu	*i*Pr	1-naphthyl	*i*Bu	1.8 ± 0.63
51	*i*Bu	*i*Pr	1-naphthyl	*i*Pr	2.3 ± 0.57

ly. In addition, aromatic substituents at the R^2 position generally led to an increase in activity when compared to their bis-isopropoxy counterparts. When the R^2 and R^3 positions are both substituted with aromatic rings (**47**, **48**), a decrease in activity was observed compared to those biphenyls with just one aromatic substituent (**46**, **50**, **51**). Possibly the increased steric bulk of two consecutive ring systems was the reason for this decreased activity. A similar phenomenon might also have caused the inactivity of **49**, which had aromatic groups at the R^1 and R^3 positions. The greatest improvement in activity was seen in those biphenyls in which the R^3 substituent was a naphthyl group, including **46**, **50**, and **51**, of which **50** was found to inhibit the Bcl-x_L/Bak interaction with a K_i value of 1.8 μM. Interestingly, the substitution patterns seen in these more active biphenyls resemble the substituents of the best terphenyl inhibitor.[15]

To probe thermodynamically the interaction between **50** and Bcl-x_L, and verify the binding affinities observed by FP, isothermal titration calorimetry (ITC) experiments were conducted. Solutions of 1.0 mM **50** were titrated into 30 μM Bcl-x_L until binding saturation was attained (Figure 4). These experiments were repeated in triplicate and each was reference subtracted. In each case, the data were fit by using a one-site binding model, and the thermodynamic constants obtained for binding are shown in Figure 4. The overall K_d for the interaction of **50** with Bcl-x_L was (7.1 ± 2.0) μM, corresponding to a favorable Gibbs free energy (ΔG) of (−7.0 ± 0.2) kcal mol^{-1}. ITC experiments showed that this interaction was governed both by favorable enthalpic (ΔH = (−5.3 ± 0.7) kcal mol^{-1}) and entropic ($T\Delta S$ = (1.7 ± 0.9) kcal mol^{-1}) contributions. The dissociation constant values obtained by ITC were in good agreement with those determined by FP. In addition, a second biphenyl **19**, which was inactive by FP, showed no affinity for Bcl-x_L in the ITC experiments. This result further demonstrates the importance of specific R-group substitution for the activity observed in this series of biphenyl compounds.

Lastly, to confirm that our compounds' mode of action involved binding to the Bak binding site of Bcl-xL, NMR experiments were conducted with **50** and Bcl-x_L. Figure 5 is based upon the HSQC chemical-shift perturbation and the peak-intensity changes of Bcl-x_L residues in the presence of **50**. Both the peak-intensity decrease and the chemical-shift change indicate ligand binding on the face of Bcl-x_L normally occupied by the Bak peptide. The mimetic appears to modulate most significantly the chemical environment of Bcl-x_L residues that occupy a ridge adjacent to the Bak binding site, as well as some residues in the Bak binding site itself. This result further supports the ability of inhibitor **50** to bind Bcl-x_L in a manner that would disrupt Bak helix binding as observed in FP displacement experiments.



#	ΔG [kcal mol^{-1}]	ΔH [kcal mol^{-1}]	$T\Delta S$ [kcal mol^{-1}]	ΔS [kcal mol^{-1} K^{-1}]	K_d [μM]
50	−7.0 ± 0.2	−5.3 ± 0.7	1.7 ± 0.9	5.7 ± 2.9	7.1 ± 2.0
19	no detectable binding				

Figure 4. ITC experimental results. Experiments were conducted in triplicate at 25 °C by titrating 1.0 mM **50** (left) and **19** (right) into 30 μM Bcl-x_L. Heats of dilution were subtracted for each titration, and data were analyzed by using MicroCal Origin7.0.

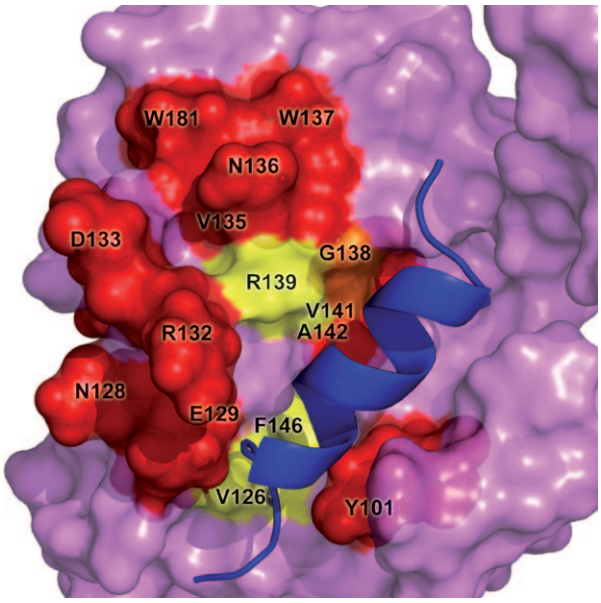


Figure 5. NMR analysis of **50** binding to Bcl-x_L. Both the peak intensity decrease (large decrease = red, moderate decrease = yellow) and the chemical-shift change (large shift = orange) indicate ligand binding on the face of Bcl-x_L normally occupied by the Bak peptide (blue).

In conclusion, we have reported the design and synthesis of a novel family of Bcl-x_L antagonists based on a biphenyl scaffold. These small-molecule inhibitors are part of a growing class of compounds that target protein–protein interactions by mimicking the spatial orientation of those residues that are important for complex formation on one of the interacting proteins. A focused library of biphenyl derivatives was prepared, and inhibition constants were determined by using an in vitro fluorescence polarization assay. The results show that the biphenyl derivatives inhibit the Bcl-x_L/Bak interaction. The best biphenyl, **50**, was found to have a K_i value of 1.8 μM by FP and a K_d of 7.1 μM, as determined by ITC. Furthermore, ^{15}N HSQC experiments confirmed that the Bak binding region of Bcl-x_L is the target area for our biphenyls. The ability of the biphenyl inhibitors to induce apoptosis in cancerous cells is currently under investigation.

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Keywords: alpha-helix mimetics · Bcl-x_L · biphenyl scaffolds · inhibitors · protein–protein interactions

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