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Submonomer synthesis of azapeptide ligands of the Insulin Receptor Tyrosine Kinase domain

Lathamol A. Kurian [†], Tammy A. Silva [†], David Sabatino [*,†]

Department of Chemistry and Biochemistry, Seton Hall University, 400 South Orange Ave., South Orange, NJ 07079, USA

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ABSTRACT

Azapeptide ligands of the Insulin Receptor Tyrosine Kinase (IRTK) were developed by solid-phase submonomer azapeptide synthesis in sufficient isolated yields (36–55%) and purities >95% for structure–activity relationship studies. The azapeptides adopted folded geometries with some proportion of random coil according to CD and NMR spectroscopy. In vitro phosphorylation of the IRTK domain in the presence of azapeptides produced a lead inhibitor, Ac-DIazaYET-NH_2 (~50% at 400 μM) whereas the [aza-DOPA^3] and [aza-Glu^4] analogs were found to stimulate IRTK phosphorylations. Thus, azapeptide ligands of the IRTK provide important modulatory activity of this important class of enzymes for anti-cancer and related applications in drug discovery.

Signal transduction of the Insulin Receptor Tyrosine Kinase (IRTK) is mediated by insulin binding to the extracellular α-subunit of the IRTK.[1] This ligand–receptor binding interaction causes a change in IRTK conformation which subsequently leads to phosphorylation of the Tyr1158, Tyr1162, Tyr1163 residues in the presence of ATP.[2] These residues are found within the activation loop of the cytoplasmic β-subunit of the IRTK domain. Once these tyrosine residues are phosphorylated, a conformational change within the activation loop occurs for additional IRTK autophosphorylation which activates the receptor for intracellular signaling activity.[3] The IRTK dictates a myriad of signaling events, such as the PI(3)K, CAP/CbI and the Ras/MAPK pathways, respectively associated with glucose metabolism, protein synthesis, and cell proliferation.[4–6] However, elevated IRTK activity has been associated with un-regulated signal transduction that uncontrollably stimulates gene expression, protein synthesis and cell division that are hallmarks of a wide range of human cancers.[7] Thus, selective regulation and potent inhibition of these types of receptors may form the basis for effective forms of cancer treatment.[8]

The pentapeptide, Ac-DIYET-NH_2 (15), derived from the activation loop of the IRTK[9] (G^{1149}DFGMTRDIY^{1158}ETDY^{1162}Y^{1163}RKGGKGL^{1170}) and specifically encompassing the autophosphorylation site (Y^{1158}) was found to inhibit full IRTK (purified from rat liver) phosphorylation to about 80% at 4 mM.[10] Moreover, competitive binding experiments in between ATP and Ac-DIYET-NH_2 (15) demonstrated that the pentapeptide was a competitive inhibitor of the IRTK domain, binding to the catalytic loop of the receptor and thereby preventing autophosphorylation of the IRTK in a non-ATP dependent manner.[10] This mechanism was also confirmed by mass spectrometry, which indicated that the Ac-DIYET-NH_2 (15) was phosphorylated by the IRTK. Other peptide ligands were developed, of which Ac-DIFET-NH_2 (16), demonstrated modest inhibition (20% at 4 mM) illustrating the importance of the Tyr side chain on peptide activity. Furthermore, computational docking simulations of the pentapeptide inhibitor (15) bound to the IRTK illustrated a folded peptide structure reminiscent of a turn conformation which may contribute to binding affinity.[10] Thus, peptide mimics (peptidomimetics) that may stabilize the putative turn conformation responsible for binding may lead to the generation of more potent IRTK inhibitors. Towards this goal, azapeptide analogs of the parent pentapeptide Ac-DIYET-NH_2 (15) were developed by submonomer solid phase synthesis to explore the importance of a turn conformation and influence of the side chain groups on IRTK inhibitory activity.

Azapeptides are a class of peptidomimetics in which one or more amino acid is replaced by an aza-residue, which substitutes the α-carbon for a nitrogen atom.[11] This substitution leads to a change in peptide backbone conformation by bending the φ and ψ dihedral angles of the aza-amino acid residue into β-turn structures as proven by CD, NMR spectroscopy, X-ray crystallography and computational analyses.[12] Moreover, azapeptide analogs of biologically active sequences have improved bioavailability, metabolic stability, receptor binding affinity and selectivity for potential therapeutic applications.[11,13]

* Corresponding author.
E-mail address: david.sabatino@shu.edu (D. Sabatino).
† These authors contributed equally.

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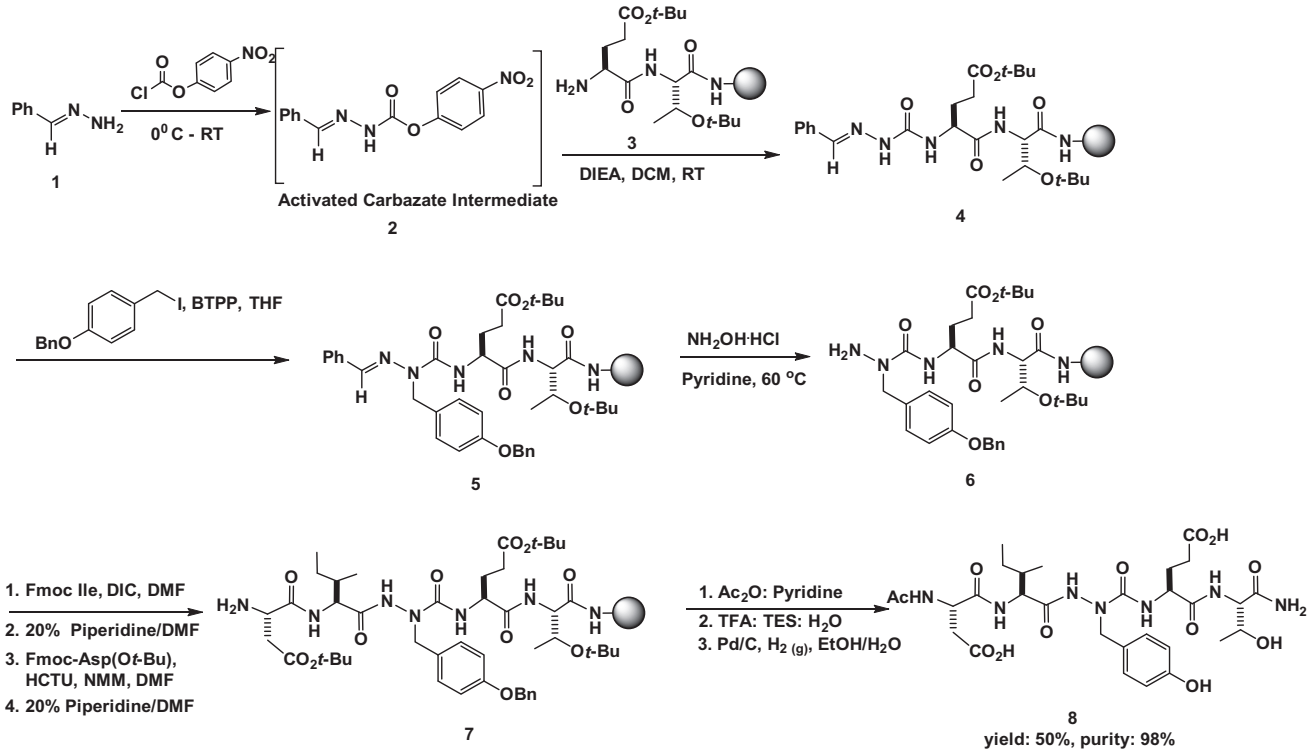
The submonomer method for azapeptide synthesis builds the aza-amino acid residue directly on solid phase, thereby facilitating the rapid generation of azapeptide libraries for structure–activity relationship studies with receptor targets.[14] For example, submonomer synthesis of GHRP-6 azapeptides has empowered scanning for biologically active turn conformations[13] and elucidated the role of the side chains that contributed to CD36 binding affinity and selectivity for anti-angiogenic and anti-atherosclerotic applications.[14] In this study, the submonomer approach was initially adopted and optimized for the preparation of Ac-DIazaYET-NH_2 (8). This methodology consisted of an activation and coupling step, which facilitated the introduction of an activated carbazate intermediate (2) onto the support-bound peptide (3) to generate the semicarbazone (4). Direct alkylation of 4-benzyloxybenzyl chloride and BTPP with semicarbazone peptide resin (4) failed to produce the desired alkylated product (5). Halogen exchange of 4-benzyloxybenzyl chloride and sodium iodide[15] produced 4-benzyloxybenzyl iodide in solution. Regioselective alkylation of the N-terminal semicarbazone bound to the peptide support (4) was next accomplished by alkylation with 4-benzyloxybenzyl iodide and the non-ionic Schwesinger phosphazene base, *tert*-butylimino-tri(pyrrolidino)phosphorane, BTPP[16] to generate the alkylated semicarbazone (5). Transamination of the N-terminal semicarbazone (5) to generate semicarbazide (6) was completed in a pre-set incubator (60 °C, 100 rpm, 12 h) by suspending the resin in a solution of hydroxylamine hydrochloride buffered in pyridine (1.5 M). Following semicarbazone deprotection, conventional solid phase peptide synthesis, SPPS,[17] was used to complete the partially protected azapeptide (7). Deprotection of the aza-Tyr benzyl group on solid phase resulted in minimal conversion (<10%) to the coveted aza-Tyr residue. Therefore, the azapeptide was cleaved from the Rink amide-linked polystyrene solid support[18] and deprotection of the acid-labile side chain protecting groups were accomplished in TFA with H_2O and TES scavenging the reactive tetrabutyl carbonium ion species.[19] The removal of the aza-Tyr benzyl protecting group was accomplished by a Pd/C catalyzed hydrogenolysis reaction in solution[20] which finally afforded the desired Ac-DIazaYET-NH_2 (8) (Scheme 1). Following purification and characterization by RP-LCMS, 8 was isolated in yields of 50% and purities >95% as ascertained by RP-LCMS.

With method in hand, azapeptide analogs of the IYE pharmacophoric region of the parent pentapeptide, Ac-DIYET-NH_2 (15) were prepared by submonomer synthesis[14] and characterized by RP-LCMS (Table 1). At the 2 position, [aza-Ile^2] and [aza-Ala^2] were introduced respectively by alkylation with 2-iodobutane and methyl iodide in the presence of BTPP, whereas the [aza-Gly^2]-residue was recovered without alkylation to generate azapeptides (9–11) in isolated yields of 51–55% and purities >95%. Of note, the aza-Ile residue was synthesized in racemic form, however, a single azapeptide diastereomer for 10 was isolated by RP-LCMS for structure–activity relationship studies. Moving to the 3 position, the [aza-Phe^3] residue was installed using KOtBu as base and benzyl bromide as alkylating reagent to eventually produce the desired Ac-DIazaFET-NH_2 (12) in good yields (43%) and isolated purities (96%). In the case of [aza-Tyr^3], and [aza-DOPA^3], halogen exchange reactions[15] converting the alkylating reagents 4-benzyloxybenzyl chloride and 3,4-dibenzyloxybenzyl chloride, respectively, to their corresponding alkyl iodides in the presence of BTPP improved yields (36–50%) and purities >95% for the desired azapeptides (8,13). Lastly, at the 4 position, [aza-Glu^4] was installed using Michael addition chemistry[14b] with KOtBu and *tert*-butyl acrylate to generate Ac-DIYazaET-NH_2 (14) in isolated yields of 42% and 96% purity.

Peptide conformation was next evaluated to determine the propensity for azapeptides to adopt the putative β-turn structure which may contribute to enhanced IRTK binding affinity (Fig. 1). In silico studies based on molecular docking simulations were initially computed to determine the binding models of the peptides on the kinase domain of the IRTK. The crystal structure of the IRTK was freely downloaded from RCSB PDB (PDB ID # 1IR3).[21] An



Scheme 1. Submonomer solid phase synthesis of Ac-DIazaYET-NH_2 (8).

Table 1
Characterization data of azapeptides synthesized in this study

	entry	R	crude purity[a]	isolated purity[b]	isolated yield[c]	mass[d]	RT (min)[e]	RT (min)[f]
Ile^2	9	–CH_3	73%	96%	51%	638.2(638.3)	7.42	5.26
	10		74%	95%	55%	680.2(680.3)	6.97	5.13
	11	–H	63%	96%	55%	624.2(624.2)	6.99	5.04
Tyr^3	8	HO–	44%	98%	50%	681.2(681.3)	9.95	6.68
	12		61%	96%	43%	666.3(666.2)	11.76	7.76
	13	HO, HO	22%	95%	36%	698.2(698.3)	8.90	6.33
Glu^4	14	HO_2C–	58%	96%	42%	680.3(680.2)	9.61	6.41

[a] Crude purity by RP-LCMS at 214 nm using 2–90% MeOH in H_2O with 0.1% FA over 15 min.
[b] Isolated purity by RP-LCMS at 214 nm using 2–90% MeOH in H_2O with 0.1% FA over 15 min.
[c] Calculated from resin loading.
[d] Observed mass (expected mass) as $[M+H]^+$ or $[M-H]^-$ by ESI-MS in positive or negative mode. Retention times (min) by RP-LCMS at 214 nm using 2–90%.
[e] Retention times (min) by RP-LC at 214 nm using 2–90% MeOH in H_2O with 0.1% FA over 15 min.
[f] Retention times (min) by RP-LC at 214 nm using 2–90% MeCN in H_2O with 0.1% FA over 15 min.

interface between the molecular graphics system PyMOL and the molecular docking suite AutoDockVina[22] was used to demonstrate the combination of docking and visualization models in between the peptides and the IRTK binding domain. A comparative binding study with the parent pentapeptide, Ac-DIYET-NH_2 (15) and the corresponding azapeptide, Ac-DIazaYET-NH_2 (8) indicated that the azapeptide (8) was positioned within a 5 Å distance from the Asp 1132 and Arg 1136 found within the catalytic loop of the IRTK (Fig. 1A). The parent peptide (15) was also found within this binding site[10], however, at distances (>5 Å) that may limit the favorable binding interactions in between the peptide and receptor (Fig. 1B). These results suggest that the peptides would prevent phosphate transfer from ATP to the autophosphorylation sites of the IRTK, thereby inhibiting its activity. Furthermore, the azapeptide turn conformation is clearly apparent within the binding site of the IRTK (Fig. 1A) and absent within the native sequence (Fig. 1B). Thus, the [aza-Tyr^3] residue is likely responsible for pre-organizing the peptide turn geometry to favor high affinity binding interactions within the catalytic loop of the kinase domain. In order to confirm the peptide structures in solution, the CD spectrum of the parent peptide Ac-DIYET-NH_2 (15), was compared to that of the azapeptide, Ac-DIazaYET-NH_2 (8) (Fig. 1C). The CD curve for the parent peptide was typical to that of a random coil or a disordered structure, characterized by the strong negative band at 190 nm.[23] Conversely, the CD curve for Ac-DIazaYET-NH_2 (8) was found to exhibit a change in conformation, characterized by the positive band near 215 nm and two negative bands near 230 and 190 nm. The observed structure is indicative of a β-turn geometry, with some proportion of random coil due to the sharp negative minima observed near 190 nm.[12,14,24] Thus, the insertion of the [aza-Tyr^3] residue within the native sequence was found to stabilize a turn conformation about the peptide backbone geometry. NMR spectroscopy was next performed to support the observed azapeptide turn conformation. The NMR spectrum of the parent peptide (15) was compared to azapeptide (8) in deuterated water and DMSO. In DMSO, the azapeptide exhibited an NMR spectrum that was consistent with a β-turn structure, with notable disappearance of the Glu NH and down-field chemical shift of the Ile NH (δ: 8.2 vs 7.6) when compared to the parent peptide (Fig. 1D and E, respectively). This observation is consistent with the expected bonding interaction found in between the carbonyl group of the Asp residue at the i position and the amino group of the Glu residue at the $i+3$ position to generate the turn conformation that is stabilized by the aza-Tyr residue at the $i+2$ position. Moreover, successive NOE correlations that are consistent with an organized azapeptide turn geometry were found within Ac-DIazaYET-NH_2 (8) but not within Ac-DIYET-NH_2 (15) whose protons appeared to be solvent exposed with fewer NOE correlations (see Supporting information).[12,13] Interestingly, azapeptides (8–14) displayed conformations that were found to vary from folded to random conformations based on the location and nature of the aza-residue in aqueous media (water and phosphate buffer) and organic (DMSO) solvents (see Supporting information) illustrating rich peptide structures that may contribute to biological activity.

A preliminary study on the inhibitory activity of the azapeptide Ac-DIazaYET-NH_2 (8) in comparison with the native sequence

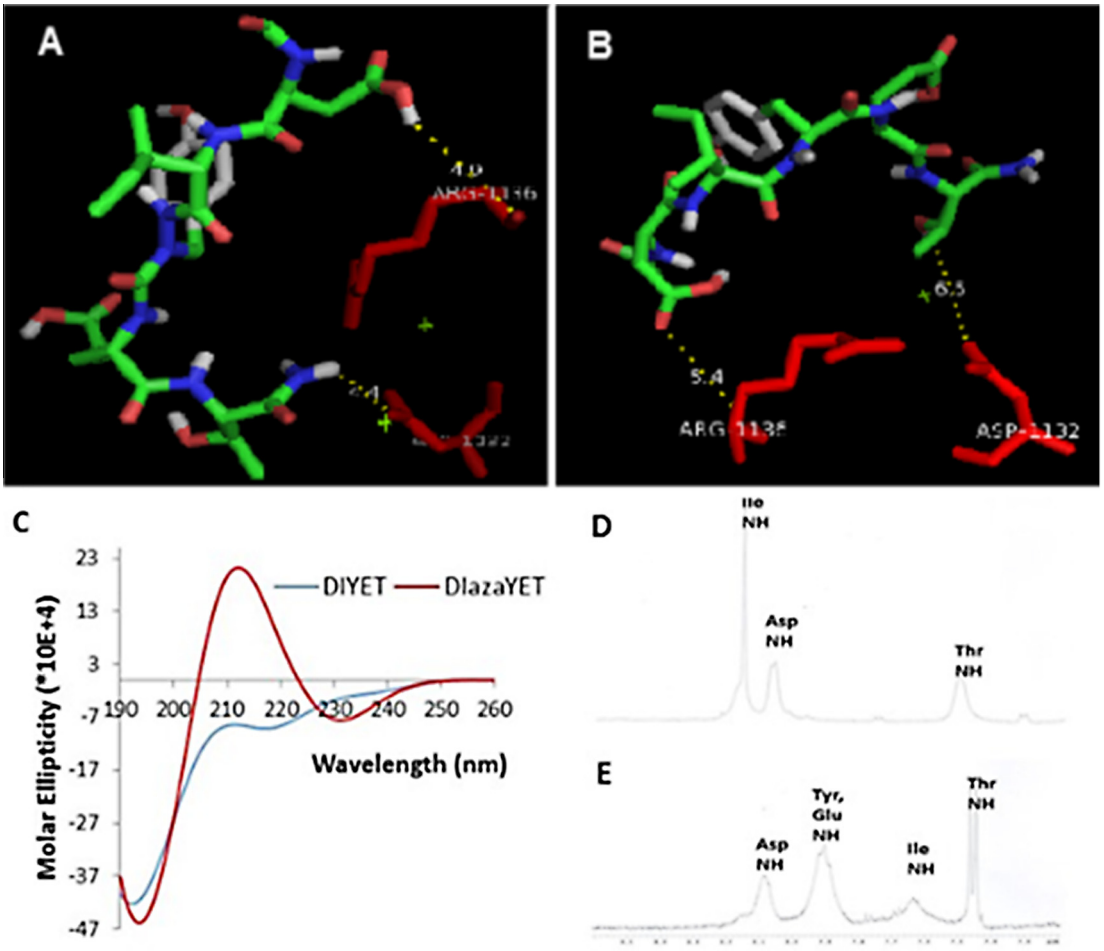


Figure 1. Structure analyses of Ac-DIYET-NH_2 (15) and Ac-DIazaYET-NH_2 (8). Molecular docking of (A) Ac-DIYET-NH_2 (15) and (B) Ac-DIazaYET-NH_2 (8) bound to the catalytic loop of the IRTK. (C) CD spectra of Ac-DIYET-NH_2 (15) and Ac-DIazaYET-NH_2 (8) (20 μM) in water. ^{1}H NMR spectra of (D) Ac-DIYET-NH_2 (15) and (E) Ac-DIazaYET-NH_2 (8) (2 mM) in DMSO-d_6.

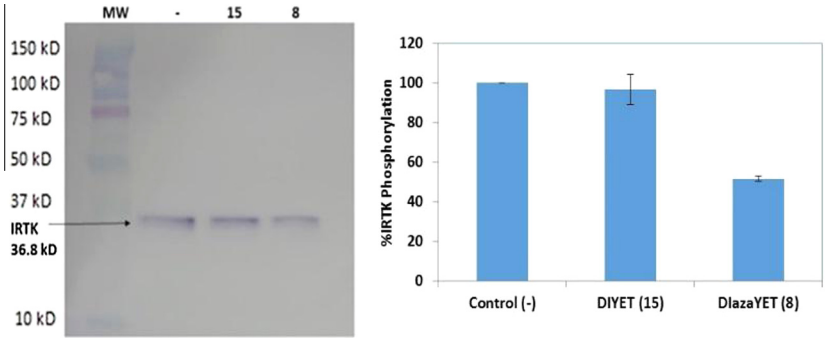


Figure 2. IRTK inhibitory activity of Ac-DIYET-NH_2 (15) and Ac-DIazaYET-NH_2 (8) at 400 μM (n = 3).

Ac-DIYET-NH_2 (15) was performed by western blot. The phosphorylation activity of the recombinant IRTK domain (residues 1005–1310) was initially determined by incubating the IRTK (200 ng/mL) and ATP (1000 μM) in a reaction buffer for 20 min at 37 °C. The reactions were quenched and the samples were then resolved by denaturing 10% Bis-Tris PAGE and transferred to a PVDF membrane for overnight blocking (2% BSA in TBST). The membrane was rinsed and treated with antiphospho-IR/IGFIR (Tyr 1158, 1162, 1163). The antigen–antibody complexes were then visualized with a goat anti-rabbit IgG antibody, Alkaline Phosphatase (AP) conjugate which catalyzed the colorimetric detection of the blue colored IRTK using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) see Supporting information. The inhibitory activity of the peptides, Ac-DIYET-NH_2 (15) versus Ac-DIazaYET-NH_2 (8), was next evaluated by conducting the IRTK phosphorylation reactions as previously described, but with the addition of peptide ligands (400 μM). In this single dose experiment, minimal inhibitory activity (<10%) was detected for the parent peptide, Ac-DIYET-NH_2 (15), whereas, Ac-DIazaYET-NH_2 (8) displayed ~50% inhibition of the IRTK autophosphorylation. In the absence of peptide, the IRTK autophosphorylation was considered to be 100% (Fig. 2). A dose–response (0–400 μM) assay was

Table 2
Percent (%) IRTK phosphorylation with peptides (400 μM) and ATP (1000 μM) ($n = 3$)

Entry	Sequence (#)	%IRTK phosphorylation
1	Ac-DIYET-NH_2 (15)	82 ± 12
2	Ac-DIFET-NH_2 (16)	84 ± 5.7
3	Ac-DazaAYET-NH_2 (9)	74 ± 19
4	Ac-DazaIYET-NH_2 (10)	73 ± 11
5	Ac-DazaGYET-NH_2 (11)	61 ± 2.3
6	Ac-DIazaYET-NH_2 (8)	40 ± 8.2
7	Ac-DIazaFET-NH_2 (12)	67 ± 9.4
8	Ac-DIaza(DOPA)ET-NH_2 (13)	128 ± 2.9
9	Ac-DIYazaET-NH_2 (14)	110 ± 3.6

next conducted, comparing the inhibitory activity of the native sequence (15) with the azapeptide (8) and a known tyrphostin (AG1024) inhibitor of the IRTK was used as control.[25] In this experiment, Ac-DIazaYET-NH_2 (8), maintained about 50% inhibitory activity of the IRTK autophosphorylations at 40 μM, albeit to a lesser extent relative to the tyrphostin inhibitor (~80% inhibition at 40 μM) and with minimal inhibitory activity (<10% at 40 μM) observed for the native sequence, Ac-DIYET-NH_2 (15), see Supporting information.

As previously described, peptides 8–16 (400 μM) were evaluated for their ability to inhibit IRTK autophosphorylations (Table 2). In this assay, the native sequences Ac-DIYET-NH_2 (15) and Ac-DIFET-NH_2 showed modest (<20%) IRTK inhibition. Meanwhile, the azapeptides (8–12) retained good inhibitory activity according to the reduced phosphorylated IRTK signals (40–74%) detected. Interestingly, the Ac-DIazaYET-NH_2 (8) analog retained the most potent inhibition (50–60%) validating the importance of the β-turn conformation on IRTK inhibitory activity. Unexpectedly, the azapeptides containing the [aza-DOPA[3]] and the [aza-Glu[4]] residues (sequences 13 and 14) stimulated IRTK autophosphorylation by 30% and 10%, respectively. Taken together, the azapeptides synthesized in this study proved to be effective modulators of IRTK phosphorylation (see Supporting information).

In sum, a new class of azapeptide ligands of the IRTK domain have been validated in this study. Submonomer solid phase synthesis furnished a small library (7) of azapeptides featuring the introduction of new aza-Ile and aza-DOPA residues. Preliminary structure–activity relationship studies demonstrated that the azapeptides adopted folded structures in solution, from which the Ac-DIazaYET-NH_2 (8) displayed a stable β-turn geometry that was found to translate to ~5-fold increase in IRTK inhibitory activity relative to the parent pentapeptide. The remaining azapeptides were found to be good inhibitors (25–60%) and also activators (10–30%) of IRTK autophosphorylations. Thus, azapeptide ligands of the IRTK may form new leads in the development of potent and selective Tyr kinase binding molecules to study their mechanisms of action for anti-cancer[7] and related applications in drug discovery,[8] all of which are a current focus of our research program.

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Supplementary data

Supplementary data (experimental procedures and characterization data of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.046.

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