New Templates for Syntheses of Ring-Fused, C^{10} β-Turn Peptidomimetics Leading to the First Reported Small-Molecule Mimic of Neurotrophin-3

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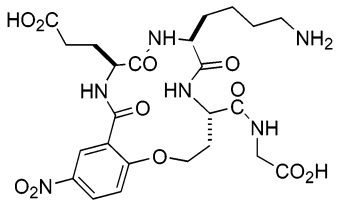
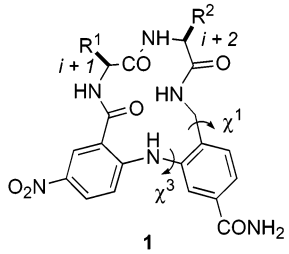
Abstract: β-Turn peptidomimetics **1** were designed to mimic hot spots of neurotrophin-3 (NT-3) and others. Solid-phase syntheses of these were developed, though limitations were encountered with scale-up. Consequently, an alternative design with **2** was investigated. **1** and **2** favored distorted type I β-turn conformations in solution. It was found that peptidomimetic **2b** has NT-3-like neurotrophic activity in cell survival assays, selectively binds the NT-3 receptor TrkC, and induces the tyrosine phosphorylation of the TrkC receptor.

Our efforts to mimic β-turn hot spots of the nerve growth factor (NGF)[1] led to the design and synthesis of several ring-fused, C^{10} β-turn analogues,[2] including compound **A**.[3] That particular mimic has since been



A



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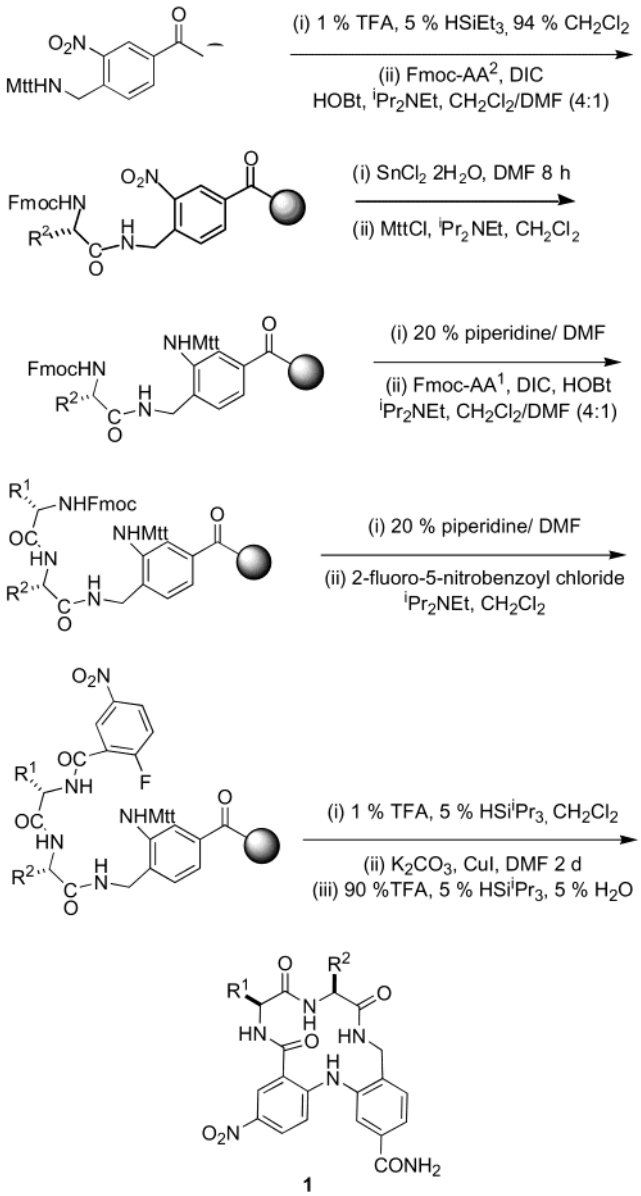
shown to bind the high-affinity NGF receptor TrkA and to enhance the response obtained from suboptimal concentrations of NGF.[3]

The next phase of this research, described here, involved two goals. The first was to design slightly less peptidic compounds that hold the key dipeptide fragment in a more rigid $(i + 1)/(i + 2)$ β-turn conformation. The second was to mimic β-turn hot spots of neurotrophin-3 (NT-3), which is a member of the neurotrophin family and related to NGF.[4] The tyrosine kinase receptor for NGF is TrkA, and the tyrosine kinase receptor for NT-3 is termed TrkC.[4] To the best of our knowledge, there have been no previous reports of a small molecule that mimics NT-3 by interacting with the TrkC receptor.

Compounds **1** were conceived after consideration of the issues outlined above. These must be more rigid than **A** and related structures because of the constraints imposed by the extra aromatic ring on the $\chi^1-\chi^3$ vectors. Consequently, practical solid-phase syntheses of these

Scheme 1



materials were required, their conformational biases in solution needed to be determined, and tests needed to be performed to determine if compounds of this type might, like **A**, elicit a functional response at one of the Trk receptors.

Scheme 1 shows how compounds **1** were constructed on Rapp HypoGel 200 RAM resin (Rapp Polymere). The most challenging, and the most interesting, step in the sequence is the penultimate one. This S_NAr macrocyclization is not particularly favorable because of the poor nucleophilicity of the arylamine. This ring closure did not occur at room temperature unless copper(+1) salts were present. Copper(+1)-mediated aryl-to-amine couplings have been known for decades but tend to involve elevated temperatures.[5] Successful coupling at room temperature in this study is therefore unusual.

Purities of crude materials isolated from the solid support are critical in syntheses such as that shown in

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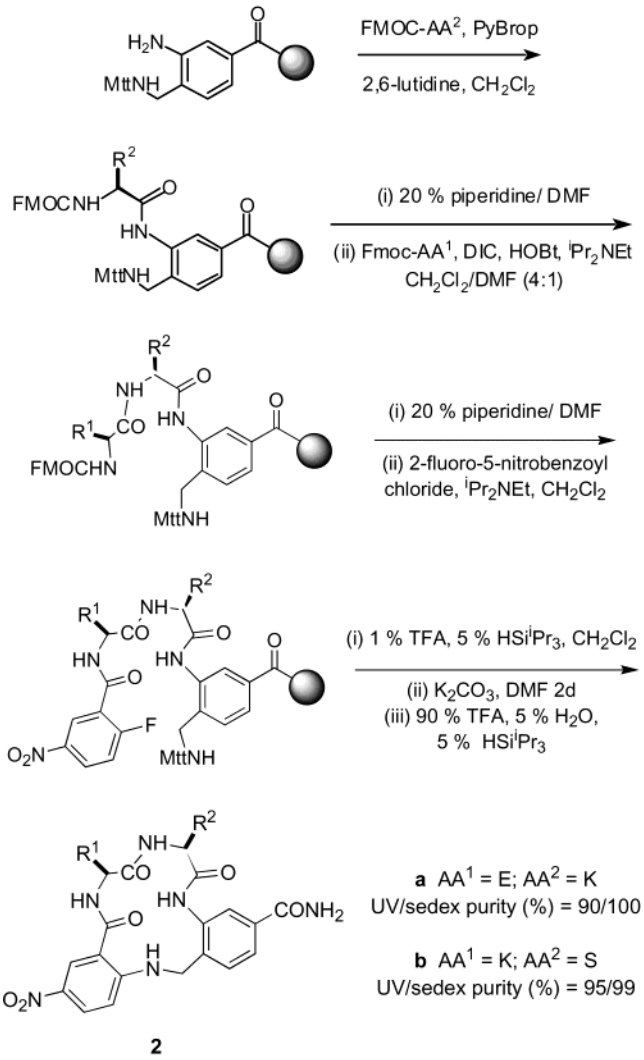
Table 1. Purities and Isolated Yields of **1**

compound	amino acids AA[1] ($i+1$)	amino acids AA[2] ($i+2$)	UV/sedex[a] purities (%)	isolated yields (%)
1a	Glu	Lys	89/99	51
1b	Lys	Ser	86/98	44
1c	Ile	Lys	81/91	40
1d	Gly	Lys	78/100	35
1e	Ser	Lys	83/97	38
1f	Thr	Lys	87/100	36
1g	Arg	Ser	74/97	48
1h	Asp	Arg	81/99	41
1i	Ile	Arg	81/98	37

[a] Sedex is evaporative light scattering detection.

Scheme 2. Synthesis of Compounds **2**



Scheme 1. Table 1 shows that the procedure works well in this regard for a number of side chain functionalities R^1 and R^2.

Methodologies that give other β-turn peptidomimetics with enhanced rigidities are also being developed. Compounds **2a** and **2b** were formed via the route outlined in Scheme 2. The scope of this approach is still being explored, but the advantages of avoiding copper catalysis are clear. Excess copper(+1) iodide, which has relatively low solubility, seems to interfere with permeation of reagents into the resin for the sequence in Scheme 1 on reasonably large scales. This is not a problem for the chemistry in Scheme 2.

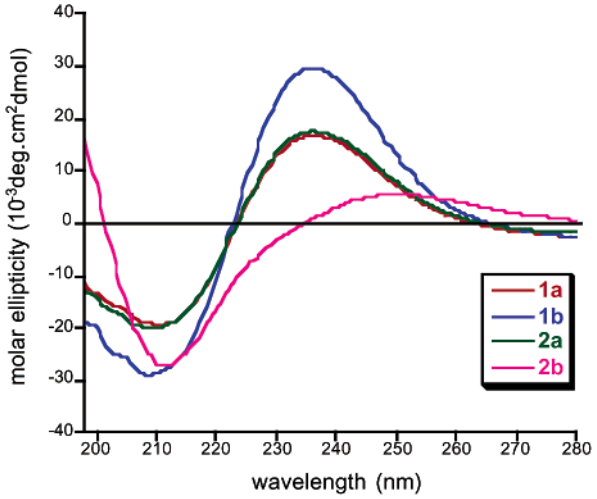


Figure 1. CD spectra of compounds **1a**,**b** and **2a**,**b** in 20% MeOH/H_2O.

Conformational preferences of peptidomimetics **1a**,**b** and **2a**,**b** were studied by experiments that included measurements of N*H* temperature coefficients, N*H*-α coupling constants, and ROESY connectivities.[6] These experimental data were then compared with virtual conformations generated via the quenched molecular dynamics technique.[7,8] Molecules such as these must populate several conformational states, but the inference from these studies is that all four compounds can adopt distorted type 1 β-turn conformations (Supporting Information). This conclusion is supported by the CD spectra of these compounds (Figure 1), which shows a minimum in the 208 nm region that is thought to be characteristic of type 1 turns.[9,10]

Cell survival screens (MTT assays) were used to access the potential of these compounds as neurotrophin analogues.[3] The choice of side chains for the compounds (Table 1) largely reflects the sequences at the NGF and/or NT-3 turn regions. This assay uses cells stably transfected with the NT-3 receptor, TrkC, or the NGF receptor, TrkA. Culture of these cells in serum-deprived conditions induces their death (0% survival). However, the cells can be rescued by addition of the appropriate concentration of neurotrophin (e.g., 2000 pM NT-3; 100% survival). Suboptimal concentrations of neurotrophin (e.g., 100 pM NT-3) affords low but significant survival (26%).

An interesting lead compound, **2b**, was identified using these cell survival assays. Peptidomimetic **2b** enhanced the survival of TrkC-expressing NIH-3T3 cells exposed to suboptimal NT-3 doses (Table 2; compare entries 8−11 with entry 3). Compound **2b** affords significant ($p < 0.05$) enhancement of cell survival when tested in combination with 100 pM NT-3 (Table 2, entries 10 and 11; 10 and 50 μM **2b**). Lower concentrations of **2b** do not afford significant cell survival enhancement (Table 2, entries 8 and 9; 0.4 and 2 μM **2b**). Compound **2b** had no effect on the survival of TrkA-expressing NIH-3T3 cells with or without NGF (Supporting Information), indicating a selective trophic effect toward TrkC. All the other compounds listed in Table 1 had no significant effect on the survival of TrkC-expressing cells.

A direct binding assay was used to access the ability of peptidomimetic **2b** to bind the Trk receptors. For that

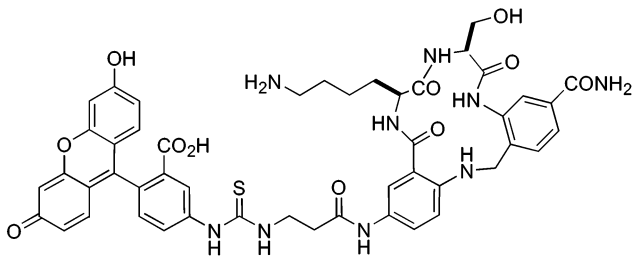
Table 2. Compound **2b** Affords Partial NT-3-Like Survival Activity in Synergy with Suboptimal (100 pM) NT-3 Concentrations

entry	**2b** concentrated (μM)	NT-3 concentrated (pM)	% survival of TrkC-expressing NIH-3T3 cells ± SEM
1	0	0	0 ± 0
2	0	2000	100 ± 0
3	0	100	26 ± 3.0
4	0.4	0	0.2 ± 0.5
5	2	0	0.1 ± 0.5
6	10	0	0.4 ± 0.3
7	50	0	0.2 ± 0.2
8	0.4	100	32 ± 1.8
9	2	100	33 ± 1.5
10	10	100	38 ± 2.2
11	50	100	36 ± 1.2

Table 3. Mean Channel Fluorescence Shifts Induced by FITC-Labeled Compounds in Cells Expressing the Indicated Receptor or No Neurotrophin Receptor (Wild-Type Cells), Demonstrating Direct Binding to Neurotrophin Receptors

		mean channel fluorescence (% of maximal binding)		
entry	test agent	TrkA cells	TrkC cells	wild-type cells
1	irrelevant FITC mAb (background)	10	11	11
2	20 μM FITC-**2b**	22	71	14
3	FITC-α-TrkC mAb		100	9
4	FITC-α-TrkA mAb	100		10
5	20 μM FITC-**2b** + NT-3 (2 nM)		42	
6	20 μM FITC-**2b** + NGF (2 nM)	9		
7	20 μM FITC-**2b** + α-TrkC mAb (20 nM)		40	

purpose, fluoresceinylated **2b (**or "FITC-**2b**") was prepared via a solid-phase route (Supporting Information).



fluoresceinated-2b

FITC-**2b** was tested for binding to transfected TrkC-expressing or TrkA-expressing cells in a fluorescence activated cell sorting (FACScan) assay as previously described.[3] Selective FITC-labeled anti-TrkC or anti-TrkA receptor monoclonal antibodies (mAb) were used to standardize maximal staining, an FITC-labeled irrelevant antibody was used to determine background fluorescence, and untransfected cells that do not express any neurotrophin receptor served as a negative control. In TrkC-expressing cells, a significant shift in the mean channel fluorescence (7× background) was observed, indicative of TrkC binding by FITC-**2b** (compare entry 2 with entry 1 of Table 3). This fluorescence diminished upon addition of saturating (2 nM) doses of NT-3 (entry 5), suggestive of an overlapping TrkC docking for NT-3 and FITC-**2b**. In TrkA-expressing cells, there was very low staining with FITC-**2b** (entry 2) (2× background),

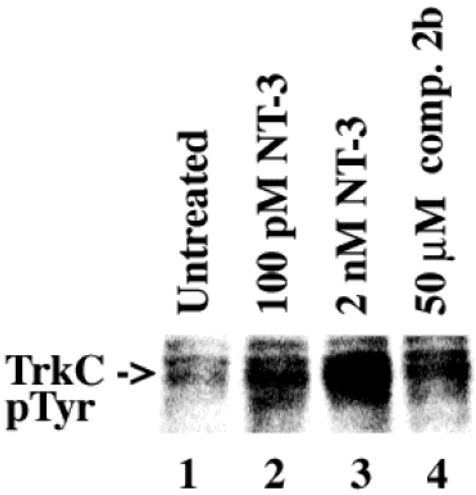


Figure 2. Western blotting assay showing that the tyrosine phosphorylation of TrkC receptors caused by **2b** at 50 μM is between that caused by 100 pM and 2 nM NT-3.

but this was significant compared to wild-type control cells that do not express neurotrophin receptors. Indeed, the fluorescent shift in the presence of saturating NGF (entry 6) was lower, suggesting specific binding of FITC-**2b** to TrkA at a docking site that may overlap with NGF. In controls, no shift above background was seen after staining wild-type cells that do not express neurotrophin receptors with FITC-**2b** or anti-receptor mAbs.

Selective binding to TrkA and TrkC suggests that **2b** behaves like NT-3, which binds both receptors but TrkA with lower affinity.[4] The fact that addition of saturating concentrations of unlabeled neurotrophin ligands {NT-3 to TrkC-cells or NGF to TrkA-cells} blocked a significant proportion of the binding of FITC-**2b** suggests that the neurotrophins and **2b** may bind to overlapping sites on the receptors. Consistent with this, an anti-TrkC monoclonal antibody that binds to the domain of TrkC where NT-3 binds also blocked the binding of FITC-**2b** (Table 3, entry 7). Cell survival assays could not be done with FITC-**2b** because its poor solubility precludes filter sterilization (FITC-**2b** is more water-insoluble than **2b**). More importantly, the presence of the solvent DMSO would interfere with cell survival assays (testing FITC-**2b** in survival assays would expose cells to 2.4% DMSO for 2−3 days, whereas in FACScan assays they are exposed to 0.9% DMSO for 1 h). However, we predict that while FITC-**2b** and **2b** are not chemically the same, they likely have similar binding and biological properties.

Tyrosine phosphorylation (pTyr) assays were performed to test if peptidomimetic **2b** would affect the intrinsic kinase activity of TrkC receptors. Such activity is implied by the enhanced cell survival data described in Table 2. Treatment with **2b** (50 μM) was compared with treatment with NT-3 (2 nM and 100 pM; positive control), with saline, and with an irrelevant peptidomimetic (negative controls; data not shown). Cells expressing TrkC were exposed for 20 min at 37 °C to the indicated treatments. Detergent extracts prepared from these cells were studied by Western blotting with anti-pTyr mAb 4G10 as previously described.[11]

Compound **2b** affords the pTyr of TrkC receptors (Figure 2). As expected, the pTyr induced by **2b** is lower

than pTyr induced by 2 nM NT-3, but it is comparable to pTyr induced by 100 pM NT-3.

It has been reported[11] that Trk receptor phosphorylation in neuronal cells can activate either a survival pathway (e.g., cell survival in serum-free media) or a differentiative pathway (e.g., neurite outgrowth). Indeed, while NGF-TrkA interactions can activate both pathways, some ligands of the TrkA receptor activate one pathway but not the other.[11,12] Compound **2b** in the absence of NT-3 induces partial TrkC tyrosine phosphorylation but does not afford cell survival; hence, we propose that it may induce the differentiative pathway. This hypothesis cannot be tested directly because DMSO (1.3% final concentration to which the cells are exposed) is required for solubilizing **2b** and this interferes with the neurite outgrowth assay (but not with the cell survival assay).

Peptidomimetic **2b** is a promising lead for development of partial agonist TrkC ligands. This NT-3 peptidomimetic appears to bind TrkC at a site overlapping the NT-3 docking region. It induces the pTyr of TrkC and under certain conditions partially agonizes the TrkC receptor to afford cell survival under apoptotic conditions. We believe that it is the first reported small-molecule mimic of NT-3 that acts on the TrkC receptor. NT-3 failed clinical trials because of short half-life (seconds to minutes), so a low efficacy but stable NT-3 peptidomimetic such as **2b** may be useful.

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Supporting Information Available: Experimental procedures for the preparation of **1** and **2**, tabulated spectroscopic data, details of the conformational analyses, and experimental procedure and scheme outlining the synthesis of FITC-**2b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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