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TrkA kinase inhibitors from a library of modified and isosteric Staurosporine aglycone

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ABSTRACT

An immobilized Staurosporine aglycone isostere where one of the indole nitrogen atoms was replaced by carbon has been sequentially functionalized to generate compounds inhibiting TrkA kinase. In the first phase, initial screening of a library of C13-hydroxymethyl-7-oxo-indenopyrrolocarbazoles resulted in several potent compounds, one of which was further optimized to generate the corresponding carbamates on solid phase. Some of the major carbamate diastereomers were found to be several-fold more potent than their alcohol parents. Synthesis, SAR analysis, kinase selectivity, and anti-tumor properties of a TrkA inhibitor (**12a**) are discussed.

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Much is known regarding the aberrant expression of nerve growth factor (NGF) and its high affinity receptor TrkA in various tumor cells, which have been suggested to be linked to progression of different human cancers such as prostate,[1] pancreatic,[2] ovarian,[3] thyroid,[4] breast,[5] and lung adenocarcinomas.[6] The TrkA family consists of three receptor kinases—TrkA, TrkB, and TrkC which can be activated by different neurotrophine class of proteins including NGF.[7] Activation of the TrkA signal transduction pathway by the prototypical neurotrophic factor, NGF, involves receptor oligomerization followed by a distinct intracellular signaling cascade leading to phosphorylation of signaling components such as PI3, PLC-γ, ras, and raf/MEK/Erk1.[8] This abnormal stimulation of NGF-TrkA contributes both to survival and proliferation of different human cancers.[7] Therefore, small molecule-based inhibitors of TrkA could disrupt the TrkA signaling pathway and find therapeutic utility against those diseases.

Unlike other protein kinase research areas,[9] the design of TrkA inhibitors is relatively less well studied. Only a few classes of inhibitors are reported in the literature.[10] The indolocarbazole-based natural products belonging to the Staurosporine (**1**) subclass are well known ATP competitive kinase inhibitors.[11] TrkA kinase inhibitors from this group of natural products include K-252a (**2**).[1b] More importantly its modified analog CEP-701 (**3**) has shown promise against prostate and pancreatic cancers[12] and is in clinical trials.[13] The principal structural difference between the Staurosporine class and other indolocarbazoles is that the former class bears an atypical bridging of both indole nitrogens in the aglycone **1a** by a single glycosyl moiety. This molecular architecture in conjunction with the distal lactam unit, which is known to interact in the kinase hinge region,[11c] may make these compounds more biased toward inhibiting a wide range of kinases. In a program to design scaffolds with greater selectivity,[11d] we reported[14a] the immobilization of an isosteric aglycone (**4a**,[14b,c] Fig. 1) of the natural product K-252a and subsequent library building strategy to create structurally diverse molecules.

In this letter, we report the chemical manipulation of the modified and immobilized aglycone **4b** (7-oxo-indenopyrrolocarbazole) to determine the structural requirements for the generation of highly potent TrkA kinase inhibitors. Kinase selectivity data and anti-tumor properties are also discussed for a compound selected for in vivo proof of concept studies.

Carbanion formation from indene **4b** was utilized in a parallel synthetic strategy to prepare compounds of types **5**–**6** (Fig. 1) using aldehydes, ketones, and epoxides as electrophiles.[14a] Since the lactam unit in the natural products is important for maintaining a hydrogen bond donor–acceptor network in the hinge region of the ATP binding pocket,[11c] the essential lactam NH of **4a** was therefore protected as a part of the resin attachment site (**4b**) in our library building strategy. Table 1 summarizes the SAR for TrkA inhibition of substituted C13-hydroxymethyl indenopyrrolocarbazoles (**5**, the aldehyde/ketone adducts of **4a**) prepared by this method.[15a,b] Where the TrkA IC_{50} values were <50 nM, the cellular inhibition scores[15c] were subsequently determined (Table 1). To facilitate rapid identification of the structural elements responsible for potent TrkA inhibition in this library, diastereomer mixtures were tested directly without further separation.[16] From this library, acetaldehyde adduct **5a** (R1 = methyl, R2 = H) was found to be a potent TrkA inhibitor (IC_{50} = 33 nM) with a cell score of 4 for inhibition of

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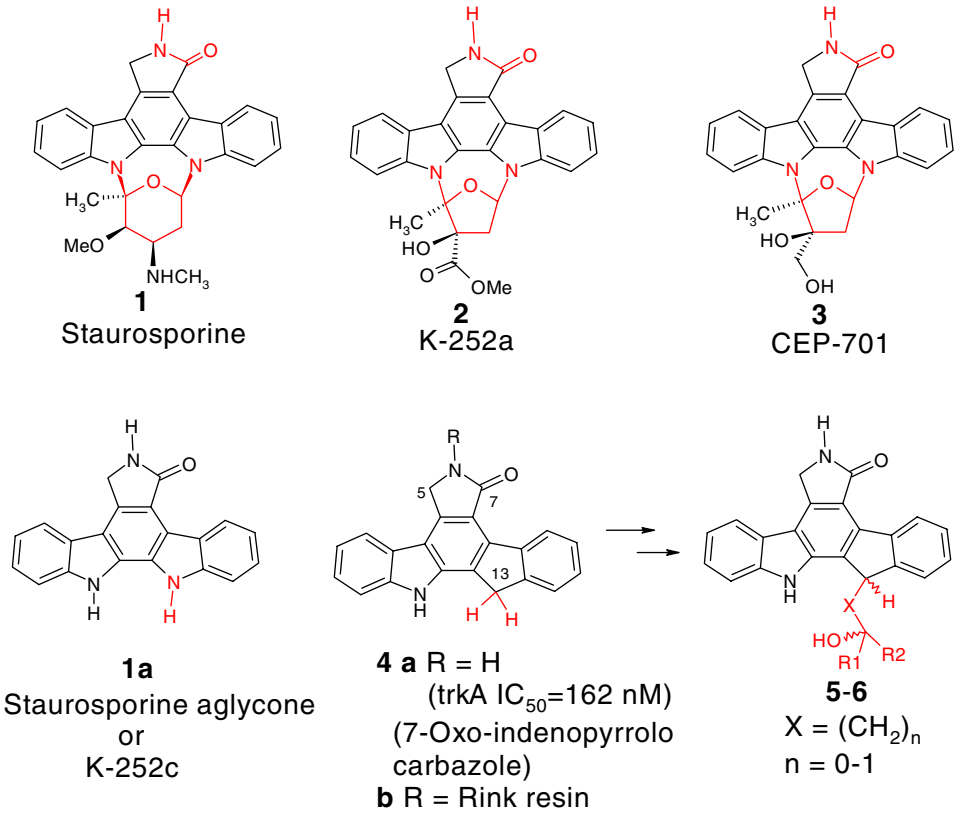


Figure 1. Natural and modified Staurosporine-like molecules.

NGF-stimulated Trk phosphorylation. This result was intriguing since **5a** was ~5-fold more potent than the parent compound (**4a**, Fig. 1) and supported our design concept that replacement of the sugar moiety in the indolocarbazole-based kinase inhibitors with new structural elements could result in potent kinase inhibitors. Increasing the size of the R1 group beyond methyl did not provide an additional increase in kinase inhibitory potency (**5b**–**f**, Table 1); heteroatom substituted alkyl chains were also not successful (**5g**–**i**). Grafting aromatic or unsaturated R1-groups (**5j**–**k**) were also detrimental for potency indicating that bulkier R1 groups were not tolerated. Ketone adducts (**5l**–**u**) were also investigated. The deuterated acetone adduct **5l** (prepared for mechanistic studies to assess effects of basicity and nucleophilicity) was also found to be active, indicating a tolerance for additional steric bulk in that region, but showed reduced cellular potency compared to acetaldehyde adduct **5a**. The SAR trend with the other ketone adducts (**5m**–**s**), where the size of the R1 group was limited to a methyl group, also resulted in potent inhibitors such as **5m** and **5n**, but without enhancement in cellular potency. Constrained cyclic ketone adducts (**5t**–**v**) were tolerated to some degree, particularly the six-membered ring analog, **5u** (IC_{50} = 72 nM).

Out of this focused chemical library, **5a** emerged as a preferred compound and was selected for further study. Like CEP-701 (**3**), K-252a (**2**), and its (3′*S*)-epi-K-252a analog, **5a** contains one hydroxyl group in the sugar region, which is believed critical for potency through hydrogen bonding interaction with the kinase.[17] Compound **6a** with a homologated carbon chain between the indene and the hydroxyl group (Fig. 2) was prepared but was found to be less potent than **5a** for TrkA inhibition. Separation of the diastereomers of **5a** by reverse phase HPLC provided the major (**7**, *RR*/*SS*) and minor (**8**, *RS*/*SR*) compounds, respectively,[18] and both compounds were found to be equipotent for inhibition of TrkA kinase.

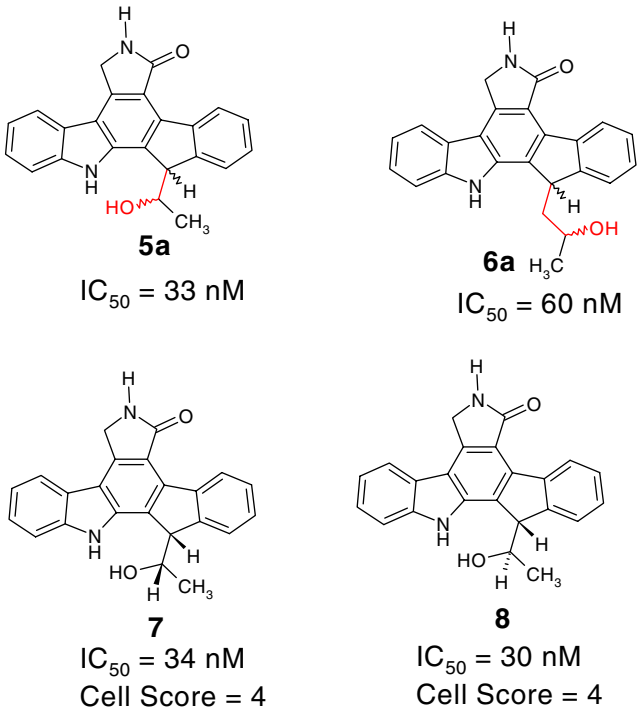
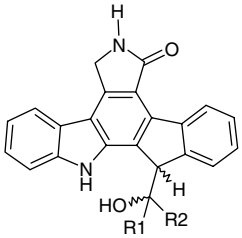


Figure 2. The diastereomers of **5a** and the epoxide adduct **6a**.

With the objective to further improve kinase potency and to achieve kinase selectivity, conversion of the hydroxyl group of **5a** to the corresponding carbamate derivatives was pursued. This modification would allow chain elongation[19] with preservation of a

Table 1
Structure–activity relationship (SAR) of C13-hydroxymethyl indenopyrrolocarbazole derivatives (**5**)



5

Compound[a]	R1	R2	TrkA IC_{50} (nM[b])	Cell score[c]
5a	H	CH_3	33	4
5b	H	Isopropyl	120	–
5c	H	Cyclopropyl	187	–
5d	H	Isobutyl	366	–
5e	H	*tert*-Butyl	90	–
5f	H	Bn	199	–
5g	H	$-CH_2-S-CH_3$	209	–
5h	H	$-CH_2-CH_2-S-CH_3$	271	–
5i	H	$-CH_2OCH_2-OBn$	250	–
5j	H	Ph	>300	–
5k	H	Ethynyl	324	–
5l	CD_3	CD_3	52	3
5m	CH_3	$-CH_2-OH$	57	3
5n	CH_3	$-CH_2-OCH_3$	25	3
5o	CH_3	$-CH_2-F$	84	–
5p	CH_3	$-CH_2-Cl$	89	–
5q[d]	CH_3	$-CH_2-NEt_2$	182	–
5r	CH_3	$-(CH_2)_3CN$	114	–
5s	CH_3	$-COOCH_3$	103	–
5t		$-CH_2-CH_2-CH_2-$	>300	–
5u		$-CH_2-CH_2-O-CH_2-CH_2-$	72	–
5v		$-CH_2-CH_2-N(CH_3)-CH_2-CH_2-$	182	–

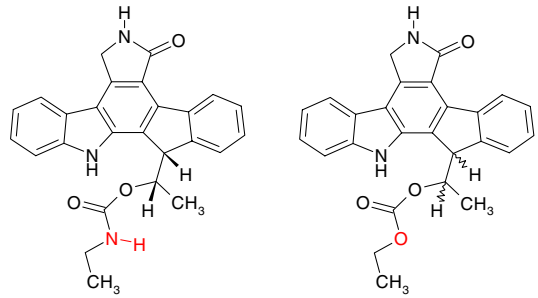
[a] Tested as mixture of diastereomers with purity ranging from 70% to 95%. See Ref. 16.
[b] For the enzyme assay see Ref. 15b.
[c] Tested at 1 μM, for the cellular assay and definition of cell scores see Ref. 15c and 15d.
[d] Tested as a TFA salt.

Table 2
Representative examples of SAR of the carbamates **12** and **13** against TrkA kinase

Compound[a]	R	IC_{50} in nM[b]	Cell score[c]
12a	Ethyl	8	4
13a	Ethyl	133	4
12b	Allyl	9	4
13b	Allyl	33	3
12c	$-CH_2-CH_2-CH_2-Cl$	14	4
13c	$-CH_2-CH_2-CH_2-Cl$	129	–
12d	$-CH_2-COOEt$	49	4
13d	$-CH_2-COOEt$	43	4
12e	Ph	>300	–
13e	Ph	>300	–
12f	Bn	52	4
13f	Bn	231	–

[a] Tested as single diastereomers with >95% purity.
[b] For the enzyme assay see Ref. 15b.
[c] Tested at 1 μM, for the cellular assay and definition of cell scores see Ref. 15c and 15d.

hydrogen bond donating group (NH). As shown in Scheme 1, the resin bound indenocarbazole (**4b**) was treated with an excess of ethyl magnesium bromide followed by trapping of the resulting carbanion with acetaldehyde to afford the immobilized compound **9**. Heating **9** with an excess of various isocyanates in toluene gave **10**,



12a
IC_{50} = 8 nM
Cell IC_{50} = 18 nM

14
(~2: 1 mixture of *RR*/*SS* and *RS*/*SR* diastereomers)
IC_{50} = 146 nM

Figure 3. The profile of the carbamate **12a** and the corresponding carbonate **14**.

Table 3
The kinase selectivity profile for **12a**

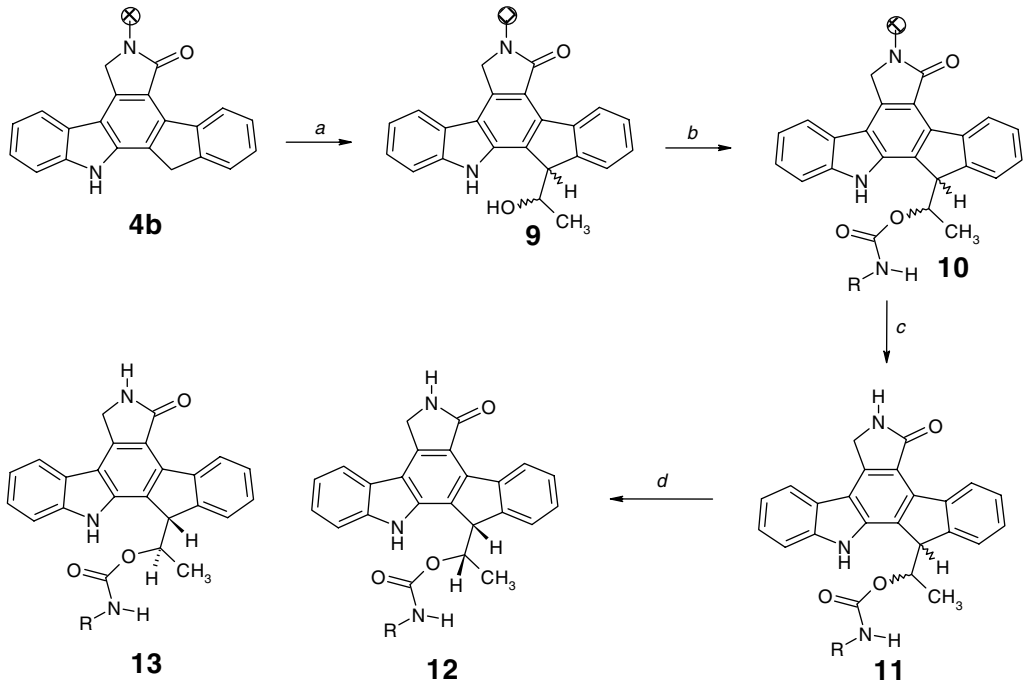
Kinase assay	% Inhibition (IC_{50} in nM)
PKC	4077
TrkA	8
FGFR	4% at 1 μM
VEGFR-2	28% at 300 nM
IRK CDK5	8% at 1 μM 4329

which upon facile deprotection with 1% TFA in dichloromethane resulted in a mixture of diastereomeric carbamates **11**. It is noteworthy to mention that the use of triethylamine base in tetrahydrofuran provided several side products including urea formation involving the indole nitrogen. In the absence of a base, the reaction proceeded cleanly with minimal byproduct formation. Separation of mixture **11** by reverse phase HPLC provided the pure diastereoisomers **12** (major, *RR*/*SS*) and **13** (minor, *RS*/*SR*). This solid phase procedure is very efficient and has been utilized successfully to make hundred milligram quantities of selected compounds.

Table 2 records the TrkA kinase inhibitory activities for a range of indenocarbazole carbamates **12** and **13**. It was very illuminating to find that the carbamate **12a** showed single digit nanomolar activity against TrkA which was ~4- and ~20-fold more potent than the parent alcohols **7** and **4a**, respectively. Interestingly, in contrast to the trend observed for the alcohols **7** and **8**, carbamate **13a** was several-fold less active than the corresponding major diastereomer **12a**. This trend in potency difference was noticed for the majority of the carbamates (Table 2). Except for carbamates bearing aryl groups (**12e** and **13e**), the major diastereomers (listed in Table 1) preserved kinase potency with acceptable cell potencies. It is also intriguing to note that although larger groups (R1 and R2) are not tolerated in **5**, the carbamates (**12**) can accommodate bigger groups. This could be due to the fact that during binding the R group in the carbamates is pointed to a different region of the TrkA protein.

Experimental data suggest that the NH group of the carbamate **12a** is critical for the enhanced binding affinity observed. The corresponding carbonate derivative (NH replaced with O) as the diastereomeric mixture (~2:1) **14** was found to be much less active (146 nM, Fig. 3) than **12a**, providing evidence for the importance of a key hydrogen bond donating group. Although the diastereomers of **14** could not be further separated, its major component displayed relative stereochemistry (*RR*/*SS*) similar to that of **12a** suggesting that the estimated IC_{50} value should be several-fold less potent than **12a**.

Compound **12a** also demonstrated excellent cellular potency (IC_{50} = 18 nM, Fig. 3). It showed a log*P* value of 4.47 and had poor



Scheme 1. Synthesis and separation of diastereomers of the carbamates (**11**). Reagents and conditions: (a) i—EtMgBr (>10 equiv), rt, THF, 45 min, ii—HMPA, 0 °C–rt, CH_3CHO; (b) toluene/isocyanate/reflux; (c) 1% TFA in dichloromethane, rt, 1 h; (d) reverse phase HPLC separation.

aqueous solubility (0.06 mg/mL). The kinase selectivity profile of the **12a** is shown in Table 3. Among a limited number kinases tested, **12a** was found to be TrkA selective.

Compound **12a** was selected as a proof of concept molecule, and tested for anti-tumor efficacy on the growth of Dunning AT-2 tumors[12c] in adult male Copenhagan rats. The compound was dosed subcutaneously (sc) at 10 mg/kg/day for 11 days. Significant inhibition in tumor growth was observed on day 11 (50% inhibition, $p < 0.05$) relative to vehicle treated controls. Compound **12a** was well tolerated by the animals, and there was no mortality or significant body weight loss observed.

In conclusion, we have identified structural features responsible for conferring potent TrkA inhibition in compounds derived from a chemical library based on the modified Staurosporine aglycone **4a** as the core structure. Acetaldehyde adduct **5a** was found to be the most potent and cell permeable compound identified in a library of compounds generated through solid phase immobilization and subsequent indene carbanion chemistry. Further derivatization of **5a** in the solid phase with different isocyanates produced very potent TrkA inhibitors (**12a** is ~4-fold more potent than **5a** and ~20-fold more potent than **4a**). Carbamate **12a** was found to be selective for TrkA among a short panel of kinases. Compound **12a** also displayed anti-tumor efficacy on the growth of Dunning AT-2 tumors in adult male Copenhagan rats after sc administration (10 mg/kg/day).

Acknowledgments

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50 mM Hepes (pH 7.4), 40 μM ATP, 10 mM $MnCl_2$, 0.1% BSA, 2.5% DMSO, and various concentrations of test compound was added to the plate. The reaction was initiated by adding recombinant human TrkA kinase and was allowed to proceed at 37 °C for 15 min. The primary antibody, N1 europium-labeled anti-phosphotyrosine (PT66) antibody (Wallac # AD0041), was then added to detect the reaction product (phosphorylated PLC-γ/GST). After 1-h incubation at 37 °C, enhancement solution (Wallac # 1244-105) was added and the plate was gently agitated. The resulting solution was then measured by time-resolved fluorescence (TRF) in the Victor[2] Multilabel Counter (Model # 1420-018). Inhibition curves for compounds were generated by plotting percent control activity versus $\log_{10}$ of the concentration of compound. IC_{50} values were calculated by nonlinear regression using the sigmoidal dose–response (variable slope) equation in GraphPad Prism.; (c) Angeles, T. S.; Lippy, J. S.; Yang, S. X. *Anal. Biochem.* **2000**, *278*, 93. *TrkA intact cell assay:* The inhibition of NGF-stimulated phosphorylation of trk was performed as described in 15c. Briefly, subconfluent NIH3T3-TrkA (positive control) and NIH3T3-vector (negative control) cells were treated with test compounds for 1 h at 37 °C. After stimulation with NGF for 5 min, the cells were rinsed with ice-cold PBS, and then lysed with lysis buffer containing 1 mM activated sodium vanadate. Clarified cell lysates corresponding to equal amounts of protein were then immunoprecipitated with anti-trk antibody (CEP-21) for 1 h, followed by incubation with Protein A–Sepharose for another hours at 4 °C. Immunoprecipitates were analyzed on a 7.5% SDS–polyacrylamide gel, and then transferred onto a Millipore PVDF membrane. The membrane was immunoblotted with 4G10 anti-phosphotyrosine antibody, followed by incubation with HRP-coupled goat anti-mouse IgG. Phosphorylated bands were visualized by enhanced chemiluminescence. Cell score as measured by a densitometer: 0 = no decrease; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%; (d) When tested at 1 μM, this subset of compounds demonstrated excellent cell permeability and inhibition of target with cell scores of 3 or 4. At 10-fold lower concentration, a difference in cellular potency of the compounds was observed with cell scores ranging from 0 to 3 (data not shown). The highest cell score at 100 nM was shown by **12a** and **12b** while the lowest cell score was obtained for **5l**, consistent with enzyme IC_{50} values (data not shown). With few exceptions (most likely due to cell permeability or solubility), a good correlation was observed between the enzyme and cell activities of the Staurosporine-like molecules described here.

16. Since the nature of the substrate controls the pi-facial selectivity for our system, diastereomeric ratios were obtained in the range of ~1:1 to 85:15. In some cases reversal in facial selectivity were also noticed (see Ref. 14a).
17. It has been shown that the (3′*S*)-epi-K-252a with an altered orientation of the free hydroxyl group of the sugar is 11-fold more potent for TrkA kinase than K-252a, indicting the importance of the orientation the hydroxyl group toward TrkA kinase activity and selectivity Gingrich, D. E.; Yang, S. X.; Gessner, G. W.; Angeles, T. S.; Hudkins, R. L. *J. Med. Chem.* **2005**, *48*, 3776.
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