Neurotrophic 3,9-Bis[(alkylthio)methyl]- and -Bis(alkoxymethyl)-K-252a Derivatives

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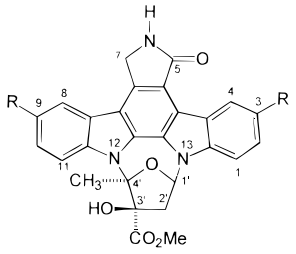
A series of 3,9-disubstituted [(alkylthio)methyl]- and (alkoxymethyl)-K-252a derivatives was synthesized with the aim of enhancing and separating the neurotrophic properties from the undesirable NGF (*trk* A kinase) and PKC inhibitory activities of K-252a. Data from this series reveal that substitution in the 3- and 9-positions of K-252a with these groups reduces *trk* A kinase inhibitory properties approximately 100- to >500-fold while maintaining or in certain cases enhancing the neurotrophic activity. From this research, 3,9-bis[(ethylthio)methyl]-K-252a (**8**) was identified as a potent and selective neurotrophic agent *in vitro* as measured by enhancement of choline acetyltransferase activity in embryonic rat spinal cord and basal forebrain cultures. Compound **8** was found to have weak kinase inhibitory activity for *trk* A, protein kinase C, protein kinase A, and myosin light chain kinase. On the basis of the *in vitro* profile, **8** was evaluated in *in vivo* models suggestive of neurological diseases. Compound **8** was active in preventing degeneration of cholinergic neurons of the nucleus basalis magnocellularis (NBM) and reduced developmentally programmed cell death (PCD) of female rat spinal nucleus of the bulbocavernosus motoneurons and embryonic chick lumbar motoneurons.

Introduction

Neurotrophism may be defined as the ability to slow or prevent compromised neurons from undergoing necrotic or apoptotic cell death and to assist compromised neurons to maintain or express a functional phenotype. The classical example of neurotrophic activity is that displayed by the protein growth factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF) which mediate neuronal survival in a variety of models *in vitro* and *in vivo*.[1] NGF has distinct, selective survival-promoting activities for cholinergic neurons in the central nervous system (CNS), as well as neurite outgrowth-promoting properties on sympathetic and sensory neurons of the dorsal root ganglia (DRG).[2] Septal forebrain cholinergic neurons respond to NGF by increasing choline acetyltransferase (ChAT) activity.[3] Insulin-like growth factor I (IGF-I), another neurotrophic factor, prevents the loss of ChAT activity in rat embryonic spinal cord cultures and reduces the programmed cell death of motoneurons *in vivo* during normal development or following axotomy or spinal transection.[4] Although positive clinical results have been reported with several neurotrophic proteins such as NGF[5] and IGF-I,[6] the therapeutic potential of these polypeptides remains limited due to their size and pharmacokinetic characteristics, which prevents their systemic administration for treatment of central neurodegenerative diseases. Clearly, the discovery and development of low molecular weight, CNS permeable molecules with neurotrophic activity represents a valuable therapeutic opportunity.

The development of small molecule therapeutics will be greatly aided by the understanding of intracellular pathways which mediate neurotrophic activity. Neurotrophic proteins bind to specific cell surface receptors, initiating intracellular signaling cascades which result in the regulation of specific gene transcription and neuronal survival.[7] Despite the initial focus on promotion of neuronal survival by protein growth factors, it is now evident that several pathways may regulate survival in a manner dependent upon the activation state of survival-promoting or death/injury-activated signaling pathways.[8] Thus, neurotrophism may result from regulation of mechanisms in addition to, and distinct from, growth factor-activated survival pathways.



1 K-252a R = H

K-252a (**1**, R = H), an indolocarbazole alkaloid isolated from *Nocardiopsis* sp.,[9] originally identified as a protein kinase C (PKC) inhibitor and subsequently found to inhibit a number of serine/threonine protein kinases,[10] has been reported to possess neurotrophic-

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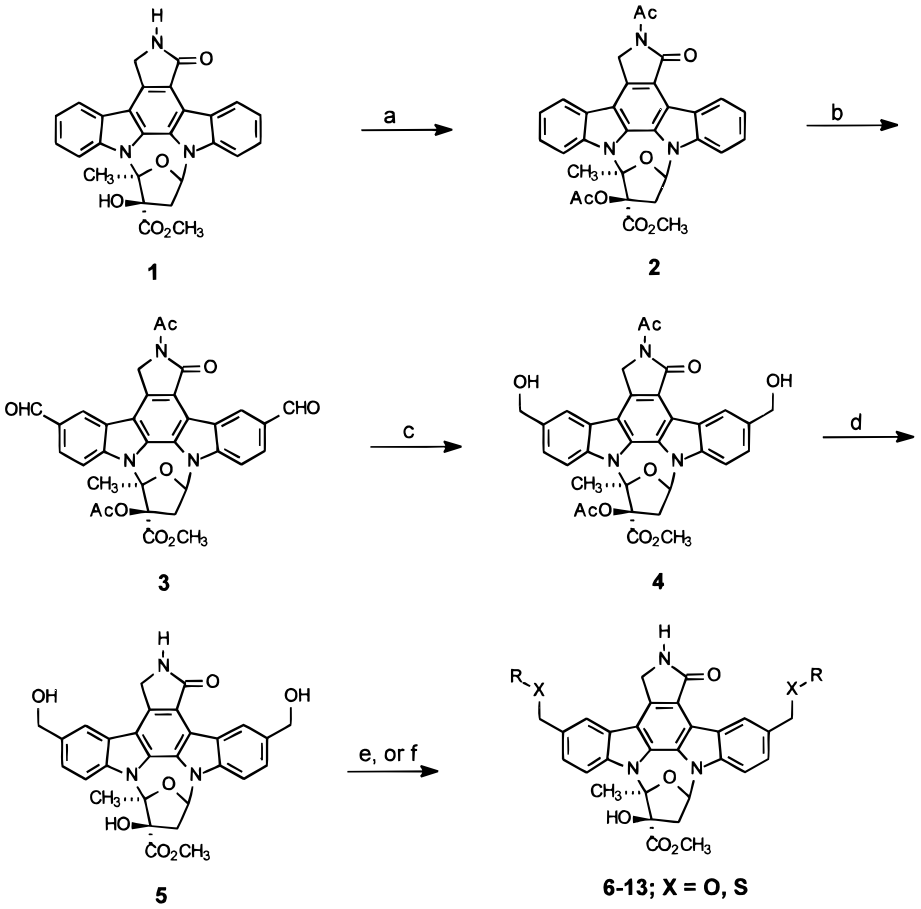
Scheme 1[a]



[a] (a) Ac_2O, DMAP, THF, room temperature, 93%; (b) Cl_2CHOCH_3, $TiCl_4$, CH_2Cl_2, 66%; (c) $NaBH_4$ CH_3OH, $CHCl_3$, 65%; (d) $NaOCH_3$, CH_3OH, $ClCH_2CH_2Cl$, room temperature, 90%; (e) ROH, CSA, CH_2Cl_2; (f) RSH, CSA, CH_2Cl_2.

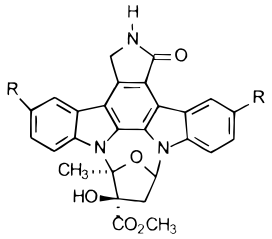
like properties.[11] K-252a has been demonstrated to promote neurite outgrowth in human SH-SY5Y neuroblastoma cells.[12] In primary cultures of embryonic neurons, K-252a induced survival of dorsal root ganglion and ciliary ganglion neurons,[13] enhanced ChAT activity in spinal cord[14] and basal forebrain cultures,[15] and promoted survival and ChAT activity in striatal cultures.[15] The neurotrophic activity demonstrated by K-252a for enhancing ChAT activity in spinal cord cultures was comparable to responses elicited by CNTF, BDNF, and IGF-I. In addition, studies have shown that K-252a protects neurons against glucose deprivation,[16] free-radical-mediated injury, and amyloid β-peptide toxicity.[17] These results suggest that the development of compounds from the K-252a class as neurotrophic agents may be effective therapy for the treatment of peripheral and central neurodegenerative diseases.

In addition to neurotrophic properties, K-252a has a number of biochemical properties which limit its utility as a therapeutic agent. K-252a inhibited NGF-induced neuronal differentiation and survival[18a−c] and specifically inhibited the autophosphorylation of the high-affinity NGF receptor *trk* A, as well as related neurotrophin receptors *trk* B, *trk* C, and *trk* oncogenes at low nanomolar concentrations.[19] A medicinal chemistry effort was undertaken to enhance and separate neurotrophic activity from the undesirable NGF (*trk* A kinase) and PKC inhibitory properties of K-252a. This paper reports on the selective neurotrophic activity of a series of 3,9-bis-(alkoxymethyl) and bis[(alkylthio)methyl] derivatives of K-252a as assessed by enhancement of ChAT activity in rat embryonic spinal cord and basal forebrain cultures, and the subsequent identification of 3,9-bis[(ethylthio)methyl]-K-252a (**8**), which exhibited greater neurotrophic potency and efficacy with low kinase inhibitory properties compared to K-252a.

Chemistry

The preparation of the 3,9-bis(alkoxymethyl)- and bis-[(alkylthio)methyl]-K-252a analogs required the synthesis of 3,9-bis(hydroxymethyl)-K-252a as the key intermediate (Scheme 1). K-252a was protected as its diacetyl derivative **2** in high yield. Diacetyl-K-252a (**2**) was converted to 3,9-dialdehyde **3** (66% yield) by treatment with 10 equiv of $TiCl_4$ and 20 equiv of α,α-dichloromethyl methyl ether in CH_2Cl_2. Reducing the quantity of α,α-dichloromethyl methyl ether in the reaction yielded higher amounts of the undesired C-3 monoaldehyde product. The position of the formyl groups was readily assigned from ^{1}H-NMR spectra. Aryl protons H-4 and H-8, which appear on **2** as a set of doublets at δ 9.09 and 8.07, respectively, appear as singlets at δ 9.53 and 8.66 on dialdehyde **3**. Diacetyldialdehyde **3** was reduced to diacetyldiol **4** (65%, $NaBH_4$, $CHCl_3$−methanol), followed by deprotection to diol **5** in 90% yield using catalytic NaOMe in methanol. Treatment of intermediate diol **5** with various alcohols or thiols in the presence of camphorsulfonic acid in CH_2Cl_2 yielded the desired bis(alkoxymethyl)- (**6**, **7**) and bis-[(alkylthio)methyl]-K-252a derivatives (**8**−**13**) in good yield.

Table 1. Neurotrophic Activity and *trk* A Inhibitory Activity of K-252a Derivatives



		% of control[a]				
		spinal cord ChAT		basal forebrain ChAT		
compound	R	30 nM	300 nM	5 0 nM	250 nM	*trk* A IC_{50} (nM)[c]
1 (K-252a)	H	<120	186 ± 3	148 ± 10	325 ± 22[d]	2.4
5	CH_2OH	<120	<120	nt[b]	nt[b]	nt[b]
6	CH_2OMe	193 ± 11	218 ± 14	168 ± 12	340 ± 21	270
7	CH_2OEt	153 ± 17	188 ± 20	<120	237 ± 22[e]	210
8	CH_2SEt	140 ± 8	280 ± 14	143 ± 15	363 ± 26	>1000
9	CH_2S^nPr	<120	315 ± 20	<120	180 ± 6	>1000
10	CH_2S^iPr	<120	289 ± 17	nt[b]	224 ± 24	>1000
11	$CH_2SCH_2CH{=}CH_2$	<120	302 ± 12	<120	191 ± 12	>1000
12	CH_2S^nBu	<120	289 ± 6	143 ± 8	200 ± 19	>1000
13	$CH_2SCH_2CH_2NMe_2$	<120	208 ± 8	<120	158 ± 15	nt[b]

[a] Enhancement of ChAT activity versus untreated control cultures. [b] nt = not tested. [c] Concentration required to inhibit 50% of *trk* kinase. [d] >200 nM concentration decreases ChAT below basal levels. [e] Maximum efficacy of 340 ± 26 at 500 nM.

Results and Discussion

The *trk* A inhibitory and neurotrophic activity of the bis-substituted K-252a derivatives (**5**−**13**) are shown in Table 1. Compounds were tested for their ability to inhibit *trk* A tyrosine kinase using an ELISA-based enzyme assay.[20] Compounds were assessed for neurotrophic activity as measured by the ability to enhance ChAT activity in embryonic rat spinal cord[14] and basal forebrain cultures.[15] ChAT catalyzes the synthesis of the neurotransmitter acetylcholine and is considered a specific biochemical marker for functional cholinergic neurons. In the spinal cord, motor neurons are cholinergic and express ChAT.[21] ChAT activity has been used extensively to study the effects of neurotrophins (e.g., NGF or NT-3) on the survival and/or function of cholinergic neurons.[2−4,14,15] During embryonic development in the rat there is a wave of motoneuron death between embryonic day 14 (E14) and E19. It is hypothesized that neuronal death occurs due to limited amounts of required trophic support and the overproduction of neurons that do not form functional synapses.[22] In spinal cord cultures (E14−E19), a significant number of cholinergic neurons would be expected to die in the absences of a motoneuron survival factor. A continual decline in ChAT activity is observed with increasing culture time.[14] In addition to spinal cord, basal forebrain neurons have also been identified as a K-252a-responsive neuronal population, showing increasing survival and ChAT activity.[15]

K-252a was used as an internal control for direct comparison in all ChAT assay experiments. The experimental data represent the mean ± standard deviation from three independent experiments. K-252a is ineffective in the spinal ChAT assay at concentrations less than 100 nM and shows a maximum enhancement of ChAT activity of 186 ± 3% above basal levels (100%) at 300 nM. In the basal forebrain assay, K-252a demonstrated good activity at 50 nM (175%) and exhibited a maximum effect on ChAT of 325 ± 22% at 200 nM. Above 200 nM, K-252a shows a decrease in ChAT activity presumably due to toxicity.[15] The history of K-252a in these assays serves as a reliable and effective reference standard. Analogs were evaluated for efficacy at 300 nM in the spinal cord and at 250 nM in the basal forebrain ChAT assays. Relative potency was assessed by screening at 30 and 50 nM in the two assays, respectively. Compounds which increased ChAT activity at least 20% above basal (120%) levels were significant and considered active.

Bis(hydroxymethyl) compound **5** was found to be inactive in the ChAT assays, whereas, in the spinal cord ChAT assay, the methyl (**6**) and ethyl (**7**) ethers demonstrated efficacy at 300 nM approximately equal to K-252a and, unlike K-252a, were effective at 30 nM. In the basal forebrain ChAT assay **6** demonstrated activity equal to that of K-252a while the ethyl derivative **7** was inactive at 50 nM; equal efficacy was observed at higher concentrations (500 nM). Similar to the ether analogs, the alkylthio derivatives retain neurotrophic activity in both the basal forebrain and spinal cord ChAT assays. The (ethylthio)methyl derivative **8** was the only sulfur analog to show activity at the lower concentrations in both the spinal cord and basal forebrain ChAT assays. Alkyl groups larger than ethyl (**8**) produced a decrease in potency in the basal forebrain and/or the spinal cord assay (see **9**−**12**). The alkyoxy and alkylthio derivatives show different structure−activity relationships based on efficacy in the spinal cord and basal forebrain ChAT models. The size of the alkylthio group does not appear to affect efficacy in the spinal cord ChAT assay, whereas alkyl groups larger than ethyl (**9**−**12**) display reduced efficacy in the basal forebrain ChAT assay. 3,9-Bis-[(alkylthio)methyl] substitution enhances efficacy over K-252a (unsubstituted R = H, 186%) in the spinal cord ChAT assay, whereas, in basal forebrain cultures, a reduced efficacy is observed except for bis[(ethylthio)-methyl] **8**, which was equal in activity to K-252a at 250 nM. As described above, K-252a shows a decrease in ChAT activity above 200 nM concentrations, presumably due to toxicity in the low-density culture[15] whereas

Table 2. Inhibitory Effect of Compound **8** on Various Kinases

kinase	IC_{50} (μM) K-252a	IC_{50} (μM) compound **8**
protein kinase C[a]	0.028	16.3
cAMP-dependent protein kinase[b]	0.016	>10
Myosin light chain kinase[c]	0.02	>10

[a] C-kinase was prepared from rat brain. [b] A-kinase was prepared from rabbit skeletal muscle. [c] MLCK was prepared from chicken gizzard.

8 did not show this inverted dose−response curve up to 2 μM (data not shown). The addition of a basic amine function (**13**) in the alkyl side chain retained neurotrophic activity only in the spinal cord ChAT assay.

The bis-substituted ether derivatives **6** (IC_{50} = 270 nM) and **7** (IC_{50} = 210 nM) were ca. 100-fold weaker than K-252a (IC_{50} = 2.4 nM) at inhibiting *trk* A tyrosine kinase, whereas in general the thio series (**8**−**12**) had IC_{50} values greater than 1 μM. These data suggest that proper substitution in the 3- and 9-positions with alkylthio groups reduces *trk* kinase inhibitory properties >500-fold while enhancing the neurotrophic activity of K-252a.

K252a has been reported to potently inhibit not only *trk* A tyrosine kinase but also additional serine/threonine kinases such as protein kinase C (PKC, IC_{50} = 28 nM), cyclic AMP dependent protein kinase (PKA, IC_{50} = 16 nM), and myosin light chain kinase (MLCK, IC_{50} = 20 nM).[10] Compound **8** was screened for inhibition of these additional serine/threonine kinases (Table 2). 3,9-Bis[(ethylthio)methyl]-K-252a (**8**) only weakly inhibited protein kinase C (IC_{50} = 16.3 μM), protein kinase A (IC_{50} > 10 μM), and myosin light chain kinase (IC_{50} > 10 μM).

The increase in ChAT activity promoted by **8** in spinal cord cultures was found associated with a promotion of motoneuronal survival in low-density spinal cord cultures enriched for motoneurons. Compound **8** which was more efficacious than K-252a in the enhancement of ChAT activity in heterogeneous spinal cord cultures was also more efficacious than K-252a in promotion of motoneuronal survival.[23a,b] At maximum effective concentrations, K-252a (150 nM) and compound **8** (300 nM) enhanced motoneuron survival 128% and 169%, respectively. The enhancement of spinal cord ChAT activity and motoneuronal survival by **8** is comparable to that elicited by optimal concentrations of protein growth factors.[23b] Again, at the higher concentrations, K-252a was cytotoxic.

Neurodegeneration, necrotic death, or loss of function of neurons (phenotypic expression) is a feature of many human neurodegenerative disorders. On the basis of its *in vitro* profile, analog **8** was further evaluated in *in vivo* models of neuronal degeneration and/or death. The enhancement of ChAT activity and motoneuronal survival in spinal cord cultures suggests potential utility in motoneuron disorders such as amyotrophic lateral sclerosis (ALS) and certain peripheral neuropathies. Approximately 50% of vertebrate motoneurons undergo programmed cell death (PCD) during embryogenesis. In the chick, 40−50% of the lumbar motoneurons die between E6 and E10.[24] To examine the effect on neuronal PCD, doses of **8** were delivered locally onto the chorioallantoic membrane surrounding the embryo from E6 through E9 and sacrificed on E10. Maximally effective doses of 2.3 and 7 μg/day/egg of **8** rescued 40% of the motoneurons that would die during this period. Lower does of 1.2 and 1.8 μg/day rescued 25%.[23b,c] Similar microgram doses of neurotrophic proteins (e.g. BDNF, IGF-I, NT-3) were also effective, with ciliary neurotrophic factor (CNTF) demonstrating the greatest efficacy (40−45%).[25] In female rats, motoneurons of the spinal nucleus of the bulbocarvernosus (SNB) die postnatal due to the absence of steroid hormone, a required non-protein survival factor. During the early prenatal life of female rats (to postnatal day (PN) 4) more than 50% of the motoneurons of the sexually dimorphic SNB are eliminated by PCD. Testicular secretion of androgen steroids reduce SNB motoneuron death in males, and when administered to females, testosterone results in a fully masculine number of SNB motoneurons.[26] Administration of **8** (1 mg/kg sc) to female rats attenuated motoneuron death with efficacy equal to that in testosterone controls, demonstrating that peripherally administered **8** was biologically active in promoting *in vivo* survival of SNB motoneurons.[23b,d] No effects on testosterone levels in rats treated with **8** were observed.

Compound **8** displayed significant effects in screens for enhancement of ChAT activity in the basal forebrain cultures. Compound **8** was assessed for neurotrophic-like activity in an *in vivo* model of basal forebrain cholinergic degeneration.[27] Basal forebrain cholinergic neurons degenerate in Alzheimer's disease (AD), and their loss may contribute to cognitive deficits of the disease. Several animal models of AD attempt to mimic basal forebrain cholinergic deficits by damaging the cortically projecting cholinergic neurons of the nucleus basalis magnocellularis (NBM). Ibotenate infusion into the NBM produced a 30% deficit in cortical ChAT activity and a 45% decrease in ChAT positive neurons in the NBM. Systemically administered **8** (0.03−1.0 mg/kg effective dose range) reduced the lesion-induced loss of cortical ChAT activity and ChAT-positive neurons to 9% and 5%, respectively. Moreover, **8** ameliorated cortical ChAT loss if administration was initiated 1 day, but not 7 days postlesion.[27] These results suggest that **8** protects cortically projecting basal forebrain cholinergic neurons from degeneration *in vivo* and that this compound may have utility in preventing the degeneration of cholinergic neurons in AD.

In conclusion, a series of 3,9-disubstituted K-252a derivatives was synthesized and evaluated for neurotrophic activity. Our data suggests that substitution with alkylthio groups in the 3- and 9-positions of K-252a reduces *trk* kinase inhibitory properties while enhancing neurotrophic activity. 3,9-Bis[(ethylthio)methyl]-K-252a (**8**) was identified as a potent neurotrophic agent *in vitro* as measured by enhancement of choline acetyltransferase activity in embryonic rat spinal cord and basal forebrain cultures. Moreover, compound **8** was found to have weak kinase inhibitory activity for *trk* A, PKC, PKA, and MLCK.

Experimental Section

Chemistry. Melting points were determined on Laboratory Devices capillary melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO IR-810 spectrometer. ^{1}H-NMR spectra were recorded on JEOL a400 (400 MHz) and Brucker AM-500 (500MHz) spectrometers. Fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS-HX110 spectrometer. Elemental analyses were performed using a Yamato MT-5 analyzer at Kyowa Hakko laboratories.

6-*N*,3′-*O*-Diacetyl-K-252a (2). To a solution of **1** (51.4 g, 110 mmol) in THF (1.1 L) were added Ac_2O (110 mL, 1.17 mol) and DMAP (67.2 g, 550 mmol). After stirring overnight, the reaction mixture was poured into 2 N HCl at 0 °C and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. Recrystallization from a mixture of MeOH−$CHCl_3$ gave 56.3 g (93%) of **2**: mp 208−210 °C; FAB-MS *m/z* 552 (MH^+); ^{1}H-NMR (500 MHz, DMSO-d_6) δ 1.71 (s, 3H), 2.22 (s, 3H), 2.24 (dd, 1H, J = 5.0, 14.7 Hz), 2.67 (s, 3H), 3.89 (dd, 1H, J = 7.5, 14.7 Hz), 3.95 (s, 3H), 5.36 (d, 1H, J = 17.7 Hz), 5.40 (d, 1H, J = 17.7 Hz), 7.30 (dd, 1H, J = 5.0, 7.5 Hz), 7.33 (m, 1H), 7.44 (m, 1H), 7.54 (m, 1H), 7.60 (m, 1H), 8.01 (d, 2H), 8.07 (d, J = 7.6, 1H), 9.09 (d, J = 7.5, 1H); HRFAB-MS calcd for $C_{31}H_{25}N_3O_7$ 552.1770, found 552.1791.

6-*N*,3′-*O*-Diacetyl-3,9-diformyl-K-252a (3). To a solution of **2** (5.00 g, 9.07 mmol) in CH_2Cl_2 (100 mL) were added $TiCl_4$ (10.0 mL, 91.2 mmol) and α,α-dichloromethyl methyl ether (16.4 mL, 181 mmol). After 2 h of stirring, the reaction mixture was poured into ice-cooled saturated $NaHCO_3$. The precipitate was filtered, and the filtrate was extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. Recrystallization from $CHCl_3$ gave 3.66 g (66%): mp 267−269 °C dec; FAB-MS *m/z* 608 (MH^+); ^{1}H-NMR (500 MHz, DMSO-d_6) δ 1.71 (s, 3H), 2.27 (s, 3H), 2.39 (dd, 1H, J = 5.0, 14.9 Hz), 2.65 (s, 3H), 3.93 (dd, 1H, J = 7.6, 14.9 Hz), 3.97 (s, 3H), 5.40 (d, 1H, J = 17.7 Hz), 5.46 (d, 1H, J = 17.7 Hz), 7.40 (dd, 1H, J = 5.0, 7.6 Hz), 8.03 (m, 1H), 8.14 (m, 1H), 8.19 (d, 1H, J = 8.8 Hz), 8.21 (d, 1H, J = 8.8 Hz), 8.66 (d, 1H, J = 1.1 Hz), 9.53 (d, 1H, J = 1.3 Hz), 10.09 (s, 1H), 10.24 (s, 1H); HRFAB-MS calcd for $C_{33}H_{25}N_3O_9$ 608.1669, found 608.1687. Anal. ($C_{33}H_{25}N_3O_9\cdot H_2O$) C, H, N.

6-*N*,3′-*O*-Diacetyl-3,9-bis(hydroxymethyl)-K-252a (4). To a suspension of **3** (10.0 g, 16.5 mmol) in MeOH-$CHCl_3$ (80 mL-500 mL) was added $NaBH_4$ (1.88 g, 49.7 mmol) at 0 °C. After 30 min of stirring at that temperature, the reaction mixture was poured into water. The resultant suspension was filtered. The precipitate was washed with water and dried *in vacuo* at 80 °C to give the crude **4** (6.53 g, 65%). **4** was used in the next step without further purification. A portion of crude **4** was purified by column chromatography (MeOH/Et_3N/$CHCl_3$, 2/0.5/98) to obtain physicochemical data: mp 297−299 °C dec; FAB-MS *m/z* 612(MH^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.72 (s, 3H), 2.21 (s, 3H), 2.22 (dd, 1H, J = 4.6, 14.6 Hz), 2.72 (s, 3H), 3.89 (dd, 1H, J = 7.2, 14.6 Hz), 3.95 (s, 3H), 4.70 (d, 2H, J = 5.1 Hz), 4.76 (d, 2H, J = 5.1 Hz), 5.22 (t, 1H, J = 5.1 Hz), 5.35 (t, 1H, J = 5.1 Hz), 5.42 (s, 2H), 7.28 (dd, 1H, J = 4.6, 7.2 Hz), 7.53 (m, 2H), 7.97 (d, 1H, J = 8.3 Hz), 7.96 (d, 1H, J = 8.8 Hz), 8.05 (s, 1H), 9.07 (d, 1H, J = 0.6 Hz); HRFAB-MS calcd for $C_{33}H_{29}N_3O_9$ 612.1983, found 612.1978. Anal. ($C_{33}H_{29}N_3O_9\cdot 1.5H_2O$) C, H, N.

3,9-Bis(hydroxymethyl)-K-252a (5). **4** (6.53 g, 10.7 mmol) was dissolved in MeOH−$ClCH_2CH_2Cl$ (100−300 mL). To the mixture was added 25 wt % NaOMe in MeOH (0.500 mL, 2.19 mmol). After 30 min of stirring min at room temperature, the reaction mixture was poured into ice-cooled water. The resultant suspension was filtered. The precipitate was washed with water and dried *in vacuo* at 80 °C to give crude **5** (5.06 g, 90%), which was used in the next reaction without further purification. A portion of crude **5** was purified by preparative TLC (MeOH/$CHCl_3$, 1/9) to obtain physicochemical data: mp 280−282 °C dec; IR (KBr) 1724, 1682 cm^{-1}; FAB-MS *m/z* 528 (MH^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.99 (dd, 1H, J = 4.9, 14.1 Hz), 2.14 (s, 3H), 3.38 (dd, 1H, J = 7.4, 14.1 Hz), 3.92 (s, 3H), 4.67 (s, 2H), 4.71 (s, 2H), 4.96 (d, 1H, J = 17.0 Hz), 5.01 (d, 1H, J = 17.0 Hz), 5.20 (br s, 2H), 7.11 (dd, 1H, J = 4.9, 7.4 Hz), 7.44−7.48 (m, 2H), 7.84 (d, 1H, J = 8.4 Hz), 7.88 (d, 1H, J = 8.7 Hz), 7.96 (s, 1H), 8.59 (s, 1H), 9.14 (d, 1H, J = 0.9 Hz); HRFAB-MS calcd for $C_{29}H_{26}N_3O_7$ 528.1770, found 528.1763. Anal. ($C_{29}H_{26}N_3O_7\cdot 1.5H_2O$) C, H, N.

General Procedure for Formation of (Alkyoxymethyl)- and [(alkylthio)methyl]- K-252a Derivatives. 3,9-Bis-[[(dimethylamino)thio]methyl]-K-252a (13). To a suspension of **5** (51.7 mg, 0.0981 mmol) and 2-(dimethylamino)-ethanethiol hydrochloride (142.9 mg, 1.01 mmol) in CH_2Cl_2 (2 mL) was added camphorsulfonic acid (206.5 mg, 0.889 mmol) at room temperature. After 4 days of stirring at room temperature, the reaction mixture was poured into saturated $NaHCO_3$ solution and extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated to give the crude product. Purification by column chromatography ($CHCl_3$/MeOH/NH_3OH, 9/1/0.1) gave 28.8 mg of **13** (42%): mp 224−226 °C dec; IR (KBr) 1732, 1670 cm^{-1}; FAB-MS *m/z* 702 (MH^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.00 (dd, 1H, J = 4.9, 14.2 Hz), 2.13 (s, 3H), 2.14 (s, 6H), 2.15 (s, 6H), 2.46−2.59 (m, 8H), 3.38 (dd, 1H, J = 7.3, 14.2 Hz), 3.92 (s, 3H), 3.95 (s, 2H), 3.98 (s, 2H), 4.95 (d, 1H, J = 17.7 Hz), 5.01 (d, 1H, J = 17.7 Hz), 6.32 (s, 1H), 7.11 (dd, 1H, J = 4.9, 7.3 Hz), 7.44−7.95 (m, 5H), 8.62 (s, 1H), 9.13 (d, 1H, J = 1.4 Hz); HRFAB-MS calcd for $C_{37}H_{43}N_5O_5S_2$ 702.2789, found 702.2773. Anal. ($C_{37}H_{43}N_5O_5S_2\cdot H_2O$) C, H, N.

Compounds **6**−**12** were prepared by the general procedure described for **13**.

3,9-Bis(methoxymethyl)-K-252a (6): mp 203−205 °C dec; FAB-MS *m/z* 555 (M^+); IR (KBr) 1732, 1663 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 2.010 (dd, 1H, J = 4.9, 14.1 Hz), 2.14 (s, 3H), 3.34 (s, 3H), 3.36 (s, 3H), 3.38 (dd, 1H, J = 7.4, 14.1 Hz), 3.93 (s, 3H), 4.58 (s, 2H), 4.62 (s, 2H), 4.98 (d, 1H, J = 16.9 Hz), 5.03 (d, 1H, J = 16.9 Hz), 6.33 (s, 1H), 7.13 (dd, 1H, J = 4.9, 7.4 Hz), 7.44−7.46 (m, 2H), 7.87 (d, 1H, J = 8.4 Hz), 7.92 (d, 1H, J = 8.7 Hz), 7.97 (d, 1H, J = 1.1 Hz), 8.61 (s, 1H), 9.17 (d, 1H, J = 1.0 Hz); HRFAB-MS calcd for $C_{31}H_{29}N_3O_7$ 555.2006, found 555.2015. Anal. ($C_{31}H_{29}N_3O_7\cdot 0.3CHCl_3$) C, H, N.

3,9-Bis(ethoxymethyl)-K-252a (7): mp 160−162 °C; IR (KBr) 1732, 1657 cm^{-1}; FAB-MS *m/z* 583 (M^+); ^{1}H-NMR (500 MHz, DMSO-d_6) δ 1.19 (t, 3H, J = 7.0 Hz), 1.20 (t, 3H, J = 7.0 Hz), 2.00 (dd, 1H, J = 4.9, 14.1 Hz), 2.14 (s, 3H), 3.39 (dd, 1H, J = 7.4, 14.1 Hz), 3.55 (q, 2H, J = 7.0 Hz), 3.56 (q, 2H, J = 7.0 Hz), 3.93 (s, 3H), 4.62 (s, 2H), 4.66 (s, 2H), 4.98 (d, 1H, J = 16.9 Hz), 5.03 (d, 1H, J = 16.9 Hz), 6.33 (s, 1H), 7.124 (dd, 1H, J = 4.9, 7.4 Hz), 7.45−7.47 (m, 2H), 7.86 (d, 1H, J = 8.3 Hz), 7.91 (d, 1H, J = 8.7 Hz), 7.97 (d, 1H, J = 1.2 Hz), 8.60 (s, 1H), 9.16 (d, 1H, J = 1.0 Hz); HRFAB-MS calcd for $C_{33}H_{33}N_3O_7$ 583.2319, found 583.2332. Anal. ($C_{33}H_{33}N_3O_7\cdot 1.5H_2O$) C, H, N.

3,9-Bis[(ethylthio)methyl]-K-252a (8): mp 163−165 °C; IR (KBr) 1725, 1680 cm^{-1}; FAB-MS *m/z* 615(M^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.23 (t, 6H, J = 7.3 Hz), 1.99 (dd, 1H, J = 4.8, 14.1 Hz), 2.132 (s, 3H), 2.489 (q, 2H, J = 7.3 Hz), 2.505 (q, 2H, J = 7.3 Hz), 3.37 (dd, 1H, J = 7.6, 14.1 Hz), 3.92 (s, 3H), 3.94 (s, 2H), 3.98 (s, 2H), 4.95 (d, 1H, J = 17.6 Hz), 5.02 (d, 1H, J = 17.6 Hz), 6.32 (s, 1H), 7.10 (dd, 1H, J = 4.8, 7.6 Hz), 7.450 (m, 2H), 7.84 (d, 1H, J = 8.5 Hz), 7.88 (d, 1H, J = 8.8 Hz), 7.95 (d, 1H, J = 1.0 Hz), 8.60 (s, 1H), 9.13 (d, 1H, J = 0.7 Hz); HRFAB-MS calcd for $C_{33}H_{33}N_3O_5S_2$ 615.1862, found 615.1869. Anal. ($C_{33}H_{33}N_3O_5S_2\cdot 0.5H_2O$) C, H, N.

3,9-Bis[(propylthio)methyl]-K-252a (9): mp 138−140 °C; IR (KBr) 1727, 1664 cm^{-1}; FAB-MS *m/z* 643 (M^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.94 (t, 3H, J = 7.3 Hz), 0.95 (t, 3H, J = 7.3 Hz), 1.56−1.66 (m, 4H), 2.00 (dd, 1H, J = 4.8, 14.1 Hz), 2.13 (s, 3H), 2.46 (t, 2H, J = 7.3 Hz), 2.47 (t, 2H, J = 7.3 Hz), 3.38 (dd, 1H, J = 7.4, 14.1 Hz), 3.92 (s, 5H), 3.96 (s, 2H), 4.95 (d, 1H, J = 17.1 Hz), 5.01 (d, 1H, J = 17.1 Hz), 6.32 (s, 1H), 7.10 (dd, 1H, J = 4.8, 7.4 Hz), 7.43−7.46 (m, 2H), 7.84 (d, 1H, J = 8.3 Hz), 7.88 (d, 1H, J = 8.6 Hz), 7.94 (d, 1H, J = 1.5 Hz), 8.60 (s, 1H), 9.12 (d, 1H, J = 1.5 Hz); HRFAB-MS calcd for $C_{35}H_{37}N_3O_5S_2$ 643.2174, found 643.2192. Anal. ($C_{35}H_{37}N_3O_5S_2\cdot 0.5CH_3OH$) C, H, N.

3,9-Bis[(isopropylthio)methyl]-K-252a (10): mp 158−160 °C; IR (KBr) 1730, 1666 cm^{-1}; FAB-MS *m/z* 643 (M^+); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.26 (d, 3H, J = 6.6 Hz), 1.27 (d, 9H, J = 6.6 Hz), 1.99 (dd, 1H, J = 5.0, 14.1 Hz), 2.13 (s, 3H), 2.88 (m, 2H), 3.38 (dd, 1H, J = 7.3, 14.1 Hz), 3.92 (s, 3H), 3.96 (s, 2H), 4.002 (s, 2H), 4.95 (d, 1H, J = 17.1 Hz), 5.02 (d, 1H, J = 17.1 Hz), 7.10 (dd, 1H, J = 5.0, 7.3 Hz), 7.44−7.47 (m, 2H), 7.83 (d, 1H, J = 8.1 Hz), 7.88 (d, 1H, J = 8.8 Hz), 7.96 (d, 1H, J = 1.7 Hz), 8.592 (s, 1H), 9.14 (d, 1H, J = 1.2 Hz); HRFAB-MS calcd for $C_{35}H_{37}N_3O_5S_2$ 643.2175, found 643.2205. Anal. ($C_{35}H_{37}N_3O_5S_2\cdot 0.5CH_3OH$) C, H, N.

3,9-Bis[(allylthio)methyl]-K-252a (11): mp 125−127 °C; IR (KBr) 1726, 1670 cm^{-1}; FAB-MS *m/z* 639 (M^+); ^{1}H-NMR

(500 MHz, DMSO-d_6) δ 2.00 (dd, 1H, J = 4.9, 14.1 Hz), 2.14 (s, 3H), 3.13 (d, 2H, J = 7.0 Hz), 3.17 (d, 2H, J = 7.1 Hz), 3.38 (dd, 1H, J = 7.4, 14.1 Hz), 3.86 (s, 2H), 3.92 (s, 2H), 3.92 (s, 3H), 4.96 (d, 1H, J = 16.8 Hz), 5.02 (d, 1H, J = 16.8 Hz), 5.16−5.19 (m, 2H), 5.22 (dd, 1H, J = 1.6, 17.0 Hz), 5.33 (dd, 1H, J = 1.5, 17.0 Hz), 5.81−5.92 (m, 2H), 6.32 (s, 1H), 7.11 (dd, 1H, J = 4.9, 7.4 Hz), 7.43−7.45 (m, 2H), 7.85 (d, 1H, J = 8.4 Hz), 7.89 (d, 1H, J = 8.7 Hz), 7.92 (d, 1H, J = 1.4 Hz), 8.60 (s, 1H), 9.12 (d, 1H, J = 1.4 Hz); HRFAB-MS calcd for $C_{35}H_{33}N_3O_5S_2$ 639.1862, found 639.1835. Anal. ($C_{35}H_{33}N_3O_5S_2 \cdot 1.4H_2O$) C, H, N.

3,9-Bis[(butylthio)methyl]-K-252a (12): mp 119−121 °C; IR (KBr) 1729, 1662 cm^{-1}; FAB-MS m/z 671 (M^+); ^{1}H-NMR (500 MHz, DMSO-d_6) δ 0.87 (t, 3H, J = 7.4 Hz), 0.88 (t, 3H, J = 7.4 Hz), 1.33−1.41 (m, 4H), 1.54−1.60 (m, 4H), 2.00 (dd, 1H, J = 4.9, 14.1 Hz), 2.13 (s, 3H), 2.48 (t, 2H, J = 7.4 Hz), 2.49 (t, 2H, J = 7.4 Hz), 3.38 (dd, 1H, J = 7.5, 14.1 Hz), 3.92 (s, 5H), 3.96 (s, 2H), 4.95 (d, 1H, J = 16.9 Hz), 5.00 (d, 1H, J = 16.9 Hz), 6.31 (s, 1H), 7.10 (dd, 1H, J = 4.9, 7.4 Hz), 7.43−7.46 (m, 2H), 7.83 (d, 1H, J = 8.4 Hz), 7.88 (d, 1H, J = 8.7 Hz), 7.94 (d, 1H, J = 1.5 Hz), 8.60 (s, 1H), 9.12 (d, 1H, J = 1.4 Hz); HRFAB-MS calcd for $C_{37}H_{41}N_3O_5S_2$ 671.2488, found 671.2494. Anal. ($C_{37}H_{41}N_3O_5S_2 \cdot 0.3H_2O$) C, H, N.

Spinal Cord ChAT Assay. The spinal cord ChAT assay was performed as previously described and references therein.[14] K-252a was used as an internal control for direct comparison in all ChAT assay experiments. The experimental data represent the mean ± standard deviation from three independent experiments. Briefly, fetal rat spinal cord cells were dissociated, and experiments were performed as described. Dissociated cells were prepared from spinal cords dissected from rats (embryonic day 14 or 15) by standard trypsin dissociation techniques. Cells were plated at 6×10^5 cells/cm^2 on poly-L-ornithine-coated plastic tissue culture wells in serum-free N2 medium supplemented with 0.05% bovine serum albumin (BSA). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 48 h. ChAT activity was measured after 2 days *in vitro*.

Basal Forebrain ChAT Assay. The basal forebrain ChAT assay was performed as previously described and references therein.[15] K-252a was used as an internal control for direct comparison in all ChAT assay experiments. The experimental data represent the mean ± standard deviation from three independent experiments. Briefly, the basal forebrain was dissected from rat embryos (day 17 or 18 embryos), and the cells were dissociated with a neutral protease (Dispas, Collaborative Research). Neurons were plated at a density of 5×10^4 cells/well (1.5×10^5 cells/cm^2) in poly-L-ornithine- and laminin-coated plates. Cells were cultured in serum-free N2 medium containing 0.05% BSA at 37 °C in a humidified atmosphere, 5% CO_2/95% air. ChAT activity was assessed 5 days after plating by using the ChAT assay as described in ref 14.

ELISA for *trk* A Tyrosine Kinase Activity. The kinase activity of baculovirus-expressed human *trk* A cytoplasmic domain was measured in an ELISA-based assay as previously described.[20] Briefly, the 96-well microtiter plate was coated with substrate solution (recombinant human phospholipase C-γ1/glutathione *S*-transferase fusion protein. Inhibition studies were performed in 100 μL assay mixtures containing 50 mM Hepes, pH 7.4, 40 μM ATP, 10 mM $MnCl_2$, 0.1% BSA, 2% DMSO, and various concentrations of inhibitor. The reaction was initiated by addition of *trk* A kinase (1 μg/mL) and allowed to proceed for 15 min at 37 °C. An antibody to phosphotyrosine (4G10) was then added, followed by a secondary enzyme-conjugated antibody, alkaline phosphatase-labeled goat anti-mouse IgG. The activity of the bound enzyme was measured *via* an amplified detection system (Gibco-BRL). Inhibition data were analyzed using the sigmoidal dose−response (variable slope) equation in GraphPad Prism.

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