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Synthesis and evaluation of 4-(1-aminoalkyl)-*N*-(4-pyridyl)-cyclohexanecarboxamides as Rho kinase inhibitors and neurite outgrowth promoters

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Abstract—The influence of stereochemistry and alkyl side chain length on the bioactivity of the Rho kinase inhibitor Y-27632 [(+)-**1**, R = Me] was examined by the synthesis of (+)- and (−)-**1**, and two alkyl chain analogs (+)- and (−)-**2** (R = *n*-propyl) and (−)-**3** (R = *n*-octyl) as well as their evaluation in enzymatic and neurite outgrowth assays.

Protein kinase inhibitors have become important targets for the development of novel therapeutics.[1] For example, Fasudil (1-(5-isoquinolinesulfonyl)-homopiperazine, Fig. 1), a Rho kinase inhibitor, has been approved for the treatment of cerebral vasospasm and is presently in clinical trials for the treatment of angina pectoris.[2] A related potent Rho kinase inhibitor, compound Y-27632 ((+)-*R*-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide, (+)-**1**, Fig. 1) has been commonly employed as a research tool.[3] Evaluation of its biological activity has shown that (+)-**1** can inhibit smooth muscle contractility, tumor cell invasion neutrophil chemotaxis, and hypertension.[4]

Rho kinase is an effector of Rho, a GTPase linked to the membrane in its active state. Recognizing the importance of Rho kinase in the Rho signaling pathway involved in axonal growth,[5] we synthesized (+)-**1** (Scheme 1) and evaluated its potential to serve as a neuroregenerative agent.[6] Inactivation of Rho kinase with (+)-**1** allowed neurons to extend axons on growth inhibitory substrates when plated on myelin in vitro.[6] Moreover, treatment of mice, after injury of their CNS, with

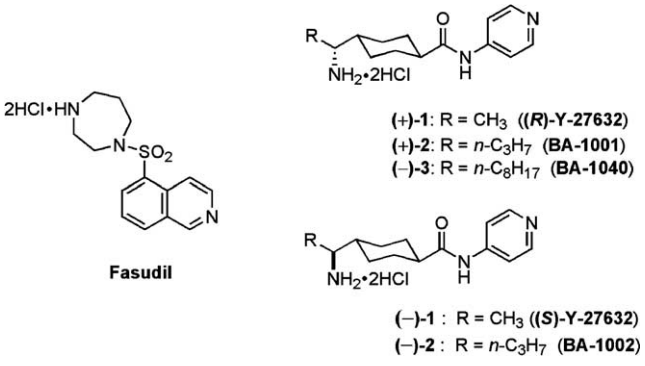

Figure 1. Representative Rho kinase inhibitors.

(+)-**1** stimulated axon regeneration and functional recovery when applied in vivo to the injured spinal cord.[6]

ε-Amino amide (+)-**1** is composed of a rigidifying cyclohexane body possessing a pyridyl amide head and amino ethyl tail. Numerous modifications of the carboxamide head group had been made; a few leading to improved activity.[7] For example, the replacement of the 4-pyridyl group by a 1*H*-pyrrolo[2,3,*b*]pyridine-4-yl heterocycle gave a 10-fold increase in inhibitor potency against Rho kinase.[3] On the other hand, there has been no

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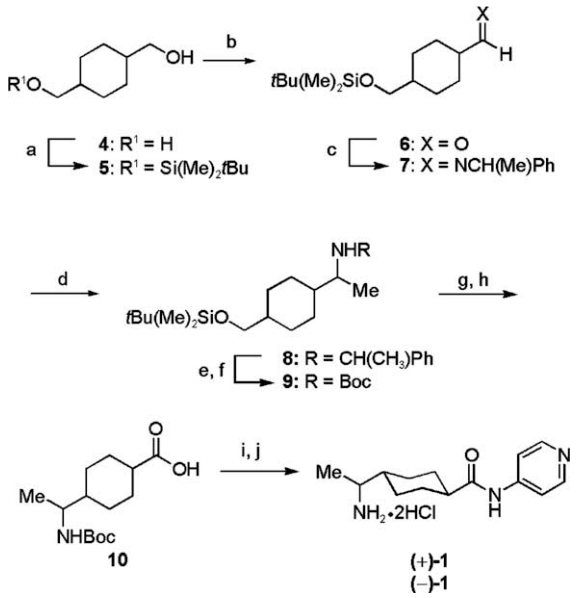


Scheme 1. Reagents and conditions: (a) TBDMS-Cl, Et_3N, DMAP, 61%; (b) PCC, Celite™, DCM, 80%; (c) (*R*)-(+) or (*S*)-(−)-phenylethylamine, $MgSO_4$, DCM; 91–96%; (d) MeMgBr, CuI, THF, −30 to −65 °C, $BF_3{\cdot}Et_2O$, 71–81%; (e) HCO_2NH_4, Pd/C, MeOH, reflux, 90–93%; (f) $(Boc)_2O$, $NaHCO_3$, Na_2CO_3, DME–H_2O (1:1), 85–94%; (g) TBAF, THF, 0 °C, 89–90%; (h) TEMPO, phosphate buffer, $NaClO_2$, NaOCl, CH_3CN, 35 °C, 68–74%; (i) 4-aminopyridine, TBTU, DIEA, DMF, rt, 79–80%; (j) HCl gas, DCM, 0 °C–rt, 73–75%.

report, to the best of our knowledge, of the influence of modification of the stereochemistry and the length of the amino alkyl chain of (+)-**1** on its kinase inhibitor activity and biology. To explore the potential of such analogs, two alternative methods have been developed for synthesizing derivatives of (+)-**1** modified at the alkyl amine.

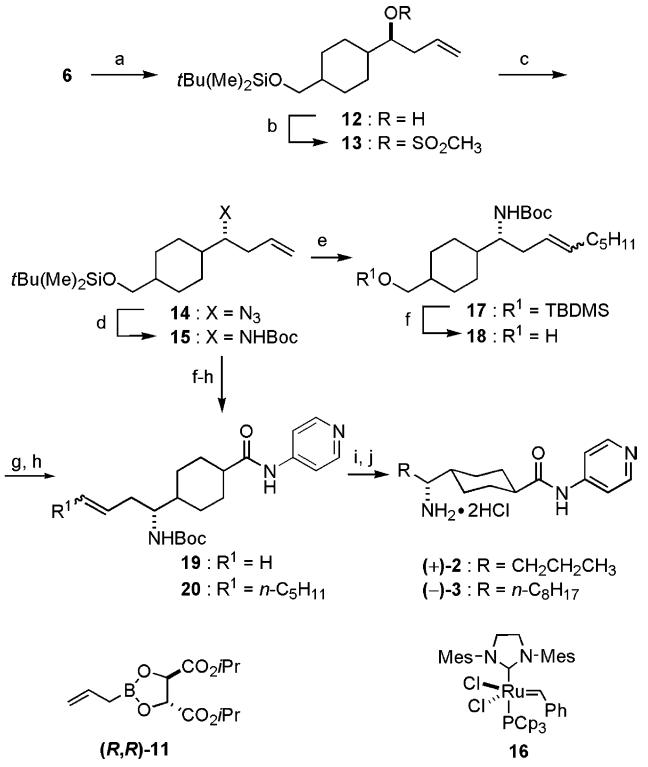
Previous syntheses of (+)-**1** and its analogs have relied on the use of α-alkylbenzylamines as chiral educts, which were subsequently acylated under Friedel–Crafts conditions at the *para*-position and reduced at the aromatic ring to provide the 1,4-disubstituted cyclohexane system.[7,8] One drawback to this approach has been the limited number of enantiomerically enriched α-alkylbenzylamines that are available commercially. Two alternative approaches to synthesize the 1,4-substituted cyclohexane system were developed as stereoselective means for generating analogs possessing a wider diversity of alkyl-branched amine moieties. In particular, the methyl group of **1** was replaced by longer alkyl chains to explore the influence of the hydrocarbon moiety on Rho kinase inhibition and neurite outgrowth promotion. Furthermore, we have evaluated the importance of the amine-bearing carbon stereochemistry on biological activity.

1,4-Cyclohexyldimethanol **4** was employed as an inexpensive starting material (Scheme 1). Selective protection of one of the hydroxyl groups as silylether **5** followed by oxidation provided aldehyde **6** in 49% overall yield (as a 70:30 mixture of *trans*:*cis* isomers by integration of the isomeric protons at 3.37 and 3.42 ppm). In one route, aldehyde **6** was condensed quantitatively with *S*- and *R*-α-methylbenzylamine in the presence of $MgSO_4$ in CH_2Cl_2 to furnish the corresponding imine, (*S*)- and (*R*)-**7**, respectively.[9] α-Methylbenzyl aldimine **7** may be reacted with a variety of nucleophilic organometallic reagents to diastereoselectively furnish different alkyl-branched amines.[10–12] For example, treatment of **7** with methyl magnesium bromide in the presence of CuI and $BF_3{\cdot}Et_2O$ in THF furnished the secondary amine **8** in >70% yield.

The newly formed center was assumed to possess the (*S*)-stereochemistry because these conditions had previously been shown to convert the (*S*)-α-methylbenzylimine of cyclohexane carboxaldehyde into the corresponding (*S*,*S*)-amine.[11] Similarly, the reaction of **7** with allyl magnesium chloride and CuI in THF gave the corresponding secondary amine in ⩾75% yield.[12] Hydrogenation of **8** using Pd/C and ammonium formate in MeOH at reflux[13] removed the chiral auxiliary and liberated the primary amine, which was protected as *tert*-butyl carbamate **9**. *N*-Boc-ε-amino acid **10** was then prepared from **9** by fluoride mediated silyl ether deprotection and oxidation of the resulting alcohol with TEMPO, $NaClO_2$, and NaOCl.[14] Amino acid **10** was coupled to 4-aminopyridine using TBTU[15] and DIEA in DMF to provide selectively the *trans*-diastereoisomer, which on deprotection of the Boc group with HCl bubbles in CH_2Cl_2 afforded amino amide **1** as the bis-hydrochloride salt. The enantiomeric purity of (+)- and (−)-**1** was evaluated by coupling the amine to *N*-(*p*-toluenesulfonyl)-L-prolyl chloride[16] and examination of the resulting amides after aqueous work-up by ^1H NMR spectroscopy. Measurement of the diastereotopic signals at 3.87 and 3.97 ppm showed a 9:1 isomeric ratio and indicated that the corresponding hydrochloride salts **1** possessed an enantiomeric excess of 80%.

In an alternative route, aldehyde **6** was reacted with either (*S*,*S*)- or (*R*,*R*)-diisopropyl-2-allyl-1,3,2-dioxaborolane-4,5-dicarboxylate **11** in toluene, which provided respectively (*R*)- or (*S*)-homoallylic alcohol **12** (Scheme 2).[17] (*R*)-Azide **14** was subsequently prepared by activation of (*S*)-alcohol **12** as a methanesulfonate and displacement with inversion using sodium azide. Reduction of azide **14** with triphenylphosphine and water in THF[18] followed by Boc-protection of the resulting amine provided carbamate **15**. Olefin metathesis[19] was next used to extend the alkyl chain of carbamate **15**. Treatment of a solution of carbamate **15** and 6-dodecene in dichloromethane with ruthenium catalyst **16** provided a 75:25 *trans*:*cis* mixture of octenyl carbamate isomers **17** in 77% yield.[20] Employing a similar sequence as described for the conversion of silyl ether **9** to its corresponding amide, *N*-(Boc)amino butenyl and nonenyl analogs **15** and **17** were transformed into their respective carboxamides **19** and **20**.

Amino butyl and nonanyl analogs **2** and **3** were finally obtained by hydrogenation of the double bond with H_2 and Pd/C in MeOH and Boc group deprotection with HCl gas in MeOH to afford their bis-hydrochloride salts.[21,22] The *S*-enantiomer of **2** was prepared using the



Scheme 2. Reagents and conditions: (a) inverse addition of **6** on **11**, 4 Å molecular sieves, toluene, −78 °C, 70%; (b) MsCl, Et_3N, DCM, 0 °C–rt, 74%; (c) NaN_3, DMF, 75 °C, 79%; (d) (i) PPh_3, H_2O, THF, 45 °C; (ii) $(Boc)_2O$, $NaHCO_3$, Na_2CO_3, DME–H_2O (1:1), 53%; (e) 6-dodecene, **16**, DCM, rt, 77%; (f) TBAF, THF, 0 °C, 77–84%; (g) TEMPO, phosphate buffer, $NaClO_2$, NaOCl, CH_3CN, 35 °C, 88–92%; (h) 4-aminopyridine, TBTU, DIEA, DMF, rt, 63–72% (i) H_2, Pd/C, MeOH, rt, 80–90%; (j) HCl/MeOH, 0 °C–rt, 91–95%.

same strategy starting from (*R*)-homoallylic alcohol **12**. The extent of stereoinduction during the allyl boronation of aldehyde **6** was determined by the synthesis of diastereomeric L- and D-(toluenesulfonyl)prolylamides on removal of the Boc group of **19** with HCl gas followed by coupling to the prolyl chlorides and examination of the resulting amides after aqueous work-up by ^1H NMR spectroscopy. Measurement of the diastereotopic allylic signals at 5.05 and 5.15 and 5.72 and 5.82 ppm showed a ratio of 9:1 and indicated that the corresponding hydrochloride salts **2** and **3** both possessed an 80% enantiomeric excess.

trans-4-(1-Aminoalkyl)-*N*-(4-pyridyl)cyclohexanecarboxamides **1**–**3** were examined as inhibitors of Rho kinase and for their ability to stimulate neurite outgrowth on tissue culture treated surface in a semi-quantitative cell-based assay (Table 1). Enantiomerically enriched (80% ee) compounds **1**–**3** were tested directly without further resolution. All compounds inhibited Rho kinase activity at 10 μM by $\geqslant$ 65% relative to vehicle (DMSO) in the presence of 100 μM ATP. Compounds **1** and **2** demonstrated similar results for Rho kinase assay; (+)-**1** and (+)-**2** are more potent than (−)-**1** and (−)-**2**. The ability of compounds (+)-**1**, (−)-**1**, and (+)-**2** to promote neurite outgrowth was demonstrated relative to vehicle (5.7, 5.8, and 4-fold vehicle, respectively). To a lesser extent (1.9-fold vehicle control), compound (−)-**2** pro-

Table 1. Effect of **1**–**3** on Rho kinase activity and neurite outgrowth

Compound	R	Rho kinase activity[a] with 10 μM (% control ± SD)	Neurite outgrowth[b] at 35 μM (% ± SD)
DMSO[c]	—	100	12 ± 6
(+)-**1**	CH_3	6 ± 3	68 ± 15
(−)-**1**	CH_3	12 ± 1	70 ± 10
(+)-**2**	n-C_3H_7	15 ± 2	48 ± 7
(−)-**2**	n-C_3H_7	35 ± 2	23 ± 1
(−)-**3**	n-C_8H_{17}	16 ± 3	0
(*R*)-Y 27632[e]	—	13 ± 2[d]	82 ± 5

[a] Rho kinase assays were performed using Kinase profiler™ service from Upstate Ltd, Dundee, UK. ATP was present at 100 μM in all assays.

[b] Neurite outgrowth assay on cell culture-treated plastic. NG108-15 cells were incubated for 4 h with test sample and fixed with 4% *para*-formaldehyde and 1% glutaraldehyde. Cells were stained with 0.05% cresyl violet and counted by microscopy. % Neurite outgrowth is stated as cells with neurites longer than cell body/total cells, where total cells is 125 ± 20 cells. Results represent mean% neurite outgrowth ± standard deviation for each experiment. This assay is semi-quantitative.

[c] DMSO 0.35% used as vehicle solution.

[d] Results taken from Davies et al. (2000).[4]

[e] Y27632 purchased from Calbiochem, USA.

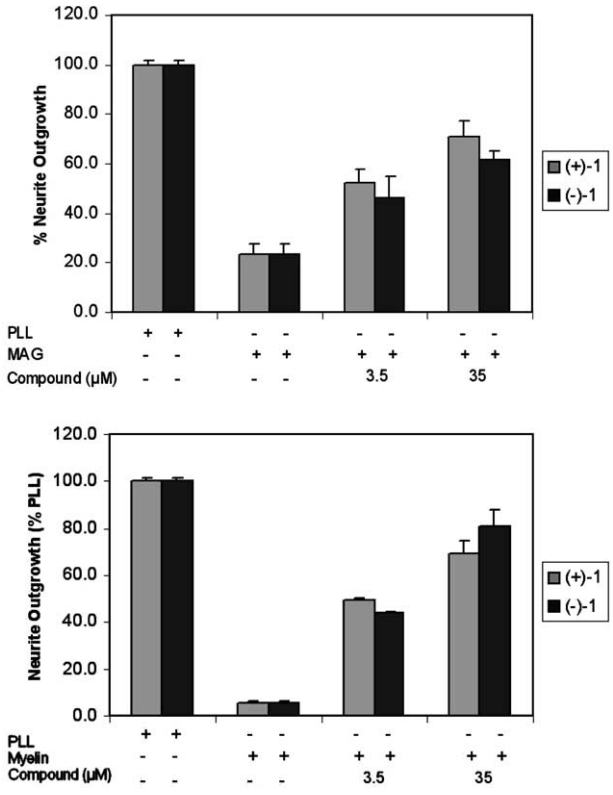


Figure 2. Neurite outgrowth assay on MAG (upper panel) and myelin (lower panel) substrates. NG108-15 cells were plated in chamber slides coated with 100 μg/mL poly-L-lysine (PLL) or with 100 μL of 80 μg/mL MAG or myelin solution, with and without compound (+)-**1** and (−)-**1** at concentrations of 0.35 and 35 μM. Cells were fixed after 24 h. Cells on MAG were stained with 0.05% cresyl violet and cells with neurites longer than 1 cell body diameter were counted. Cells on myelin were counted as described in Dergham et al. 2002.[6]

moted neurite outgrowth. Although compound (−)-**3** demonstrated a significant effect on Rho kinase activity, it failed to promote neurite outgrowth and caused cell rounding instead.

The ability of compounds (+)- and (−)-**1** to promote neurite outgrowth was examined on two inhibitory substrates, myelin-associated glycoprotein (MAG) and myelin (Fig. 2). After 24 h on MAG or myelin, few NG108-15 cells showed neurites. However, following treatment with either (+)- or (−)-**1**, the percentage of NG108-15 cells with neurites significantly increased as compared to untreated cells plated on MAG or myelin. Both (+)- and (−)-**1** helped overcome the inhibitory effect of MAG and myelin on neurite outgrowth promotion.

Stereochemistry at the amine-bearing center had a limited influence on the biological activity of compounds **1** and **2**; both enantiomers possessed the ability to inhibit Rho kinase activity and promote neurite outgrowth. In contrast, the octyl analog (−)-**3** did not stimulate neurite outgrowth and induced cell rounding, suggesting cell toxicity. Although no clear relationship between Rho kinase inhibition and neurite outgrowth promotion was established, several Rho kinase inhibitors did promote neurite outgrowth. Based on the influences of modifications of the stereochemistry and alkyl chain length of compounds **1**–**3**, opportunity may exist to modify these features to create cell permeable compounds with improved activity.

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20. The isomeric ratio 75:25 *trans*:*cis* was assigned by integration of isomeric signals at 5.26 and 5.35 and 5.47 and 5.57 ppm. The major isomer was assigned the *trans* isomer based on the 15.3 Hz coupling constant for the olefin protons.
21. In the case of **2** the Boc group was removed before hydrogenation; with **3** the hydrogenation preceded Boc deprotection.
22. The physical properties for **1** were the same as observed in Ref. 5: ^{13}C NMR (100 MHz, CD_3OD) δ 178.2, 155.5, 143.1, 115.8, 53.1, 46.6, 41.7, 29.5 (2C), 28.9, 27.6, 15.9.
Specific rotation concentrations are reported in g/100 mL:
(+)-**2**: ^{1}H NMR (400 MHz, CD_3OD) δ 1.01 (t, 3H, $J = 7.1$ Hz), 1.25–1.65 (m, 10H), 1.88 (m, 2H), 2.10 (d, 2H, $J = 12$ Hz), 2.54 (m, 1H), 3.08 (m, 1H), 8.21 (d, 2H, $J = 7.31$ Hz), 8.60 (d, 2H, $J = 7.3$ Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 178.5, 155.9, 142.1, 114.8, 56.2, 45.7, 41.4, 39.4, 31.9, 28.7, 27.6, 27.5, 18.6, 13.2; $[\alpha]_D^{20}$ 2.7 (*c* 0.58, MeOH); HRMS calcd for $C_{16}H_{26}NO_3$ ([MH$^+$]): 276.2075. Found: 276.2079.
Compound **2**: $[\alpha]_D^{20}$ − 2.7 (*c* 0.4, MeOH); HRMS calcd for $C_{16}H_{26}NO_3$ ([MH$^+$]): 276.2075. Found: 276.2081.
Compound **3**: ^{1}H NMR (400 MHz, CD_3OD) δ 0.93 (t, 3H, $J = 7$ Hz), 1.40 (m, 15H), 1.65 (m, 5H), 1.92 (m, 2H), 2.08 (d, 2H, $J = 14$ Hz), 2.49 (m, 1H), 3.08 (m, 1H), 8.15 (d, 2H, $J = 6.5$ Hz), 8.58 (d, 2H, $J = 6.8$ Hz); $[\alpha]_D^{20}$ –21.6 (*c* 0.125, MeOH); MS (TIC) *m/z* 346.2 ([MH$^+$]).