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Rho-kinase Inhibitors: Pharmacomodulations on the Lead Compound Y-32885

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In order to specify structure–activity relationships we have synthesized new series of analogues of the Rho-kinase inhibitor (R)-(+)-*N*-(4-pyridyl)-4-(1-aminoethyl)benzamide (Y-32885). The structural modifications concerned the 1-aminoethyl, the pyridyl and the amide groups which are the main features of this lead compound. Our analogue derivatives were evaluated on GTPγS-induced contraction in permeabilized smooth-muscle and on the actin cytoskeleton. All the modifications result in a diminution or a loss of activity showing that interactions of Y-32885 with the catalytic domain of Rho-kinase seem to be particularly definite and sensitive to structural variations. The presence of a pyridine moiety and a basic amine group separated by a spacer bearing an amide function are of utmost importance.

Keywords: Rho-kinase; Smooth-muscle; Contraction; Inhibitors; Y-32885; Structure–activity relationships

INTRODUCTION

Hypercontraction or abnormal smooth-muscle contraction may be a major cause of disease states such as hypertension and asthma, and a smooth-muscle relaxant that modulates this process would be therapeutically useful. Stimulation of smooth-muscle by specific agonists induces Ca^{2+} mobilization and activation of myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC) and activates the myosin adenosine triphosphatase. This sequence of events results in contraction of smooth-muscle[1–3] and interaction of actin and myosin for stress fiber formation in non-muscle cells.[4] However, because Ca^{2+} concentration does not always parallel the degree of MLC phosphorylation and contraction, an additional important mechanism of regulation that can modulate the levels of MLC phosphorylation and degree of contraction independently of Ca^{2+}, has been proposed (the so-called Ca^{2+}-sensitization).[5] The small guanosine triphosphatase (GTPase) RhoA, a member of the Rho subfamily of the Ras superfamily of monomeric GTPases, plays a major role in Ca^{2+}-sensitization.[6,7] In permeabilized smooth muscle cells, the non-hydrolysable GTP analog, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), increases MLC phosphorylation at submaximal Ca^{2+} concentrations through activation of RhoA.[8] RhoA is converted from the inactive guanosine diphosphate-bound form to the active GTP-bound form,[9] in response to various agonists whose receptors are coupled to heterotrimeric G proteins such as lysophosphatidic acid (LPA).[10] RhoA-GTP binds to specific effectors and then exerts its biological functions which include regulation of stress fiber and focal adhesion formation, cell morphology, cell aggregation, cell motility, cytokinesis, membrane ruffling, neurite retraction, microvilli formation and smooth muscle contraction.[11–13]

Among the isolated Rho effectors, Rho-kinase/ROKα/ROCK II,[14,15] a Ser/Thr protein kinase, was identified as a GTP-Rho-binding protein from bovine brain by affinity column chromatography on matrix-bound GTP-Rho.[16] ROKβ/ROCK I is an isoform of Rho-kinase.[14,17] Rho-kinase has a kinase domain in its N-terminal domain, a coiled-coil domain in its

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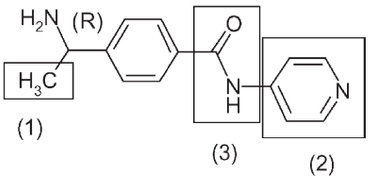


FIGURE 1 Structure of Y-32885 with the three components which have been modified.

middle portion, and a putative pleckstrin-homology (PH) domain in its C-terminal domain that is split by the insertion of a Cys-rich region. The Rho-binding (RB) domain of Rho-kinase is localized in the C-terminal portion of the coiled-coil domain,[16] and the Rho-kinase activity is enhanced by binding GTP-Rho. Rho-kinase is able to regulate the phosphorylation of MLC by the direct phosphorylation of MLC and by the inactivation of myosin phosphatase through the phosphorylation of myosin-binding subunit (MBS).[18,19] This inhibition, in turn, results in an increase in MLC phosphorylation and, consequently contributes to Rho-mediated stress fiber formation[20–22] and smooth-muscle contraction.[23]

Recently, several chemical compounds such as Y-32885 (Figure 1) have been shown to inhibit the Rho-kinase activity and exert antihypertensive effects in animal models.[24] Y-32885 is a compound containing a pyridine moiety. Since this compound inhibits Rho-kinase in a competitive manner with ATP, it may interact with the catalytic domain of Rho-kinase.[25]

In the present study, chemical modifications around the structure of Y-32885 have been carried out in order to specify structure–activity relationships and eventually to improve the pharmacological profile of this compound. These modifications affected the 1-aminoethyl group responsible for asymmetry, the pyridinyl nitrogen and the amide groups which could be involved in ionic and/or hydrogen bonds with the catalytic domain of Rho-kinase.

MATERIALS AND METHODS

Instrumentation

Melting points were determined on a BUCHI 535 apparatus and are not corrected. IR spectra were obtained in KBr pellets with a Vector 22 Bruker spectrometer. ^{1}H-NMR spectra were obtained using an AC 300 Bruker spectrometer in d_6-DMSO or $CDCl_3$ as solvent. Chemical shifts are expressed as δ values (ppm) relative to Me_4Si as internal standard. Elemental analyses for new substances were performed by CNRS Laboratories (Vernaison, France) and the results were found $\pm 0.4\%$ within the theoretical values. All reactions were monitored by thin-layer chromatography (TLC) using 0.2 mm-thick silica gel plates 60F 254 (5735 Merck).

Synthesis

General Procedure for the Preparation of Compounds 2a–b

To a solution of NaOH 1 N (20–100 mL) was added the desired aminoacid (**1a**–**b**) (6.0–33.0 mmoles), dioxane (20–100 mL) and dropwise benzyl chloroformate (0.94–5.18 mL, 6.6–36.3 mmoles) during 2–10 min. The reaction was stirred overnight at room temperature or until a reasonable conversion was reached (TLC control). The reaction mixture was washed with EtOAc. The aqueous layer was brought to pH = 1.0 with 3 N HCl and extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to give an oily residue which was triturated with petroleum ether to give a crude white solid. The obtained compounds were further purified by recrystallisation from the specified solvent. Experimental data for the compounds are reported below.

4-(Benzyloxycarbonylaminomethyl)benzoic Acid, **2a**

It was prepared using 4-(aminomethyl)benzoic acid **1a** (5.00 g, 33.0 mmoles): white crystals 4.70 g (16.5 mmoles) (50% yield), mp 194–195°C (toluene). ^{1}H-NMR (d_6-DMSO) δ 4.30 (d, 2H, $J = 6.11$ Hz, CH_2), 5.10 (s, 2H, CH_2), 7.20–7.50 (m, 7H, H_{ar}), 7.80–8.10 (m, 3H, H_{ar}; OCONH), 12.80 (signal, 1H, COOH).

4-(1-Benzyloxycarbonylaminoethyl)benzoic Acid, **2b**

It was prepared using 4-(1-aminoethyl)benzoic acid **1b** (1.00 g, 6.0 mmoles): white crystals 0.90 g (3.0 mmoles) (50% yield), mp 189–190°C (toluene). ^{1}H-NMR (d_6-DMSO) δ 1.30 (d, 3H, $J = 7.00$ Hz, CH_3), 4.75 (m, 1H, CH), 5.00 (s, 2H, CH_2), 7.25–7.40 (m, 5H, H_{ar}), 7.43 (d, 2H, $J = 8.00$ Hz, H_{ar}), 7.85–8.00 (m, 3H, H_{ar}; OCONH), 12.90 (signal, 1H, COOH).

General Procedure for the Preparation of Compounds 3a–d, 8a–c, 13

To a stirred solution of the appropriate acid (**2a**–**b**, **6a**,**b** or **12**) (3.34–34.0 mmoles) in dichloromethane (20–200 mL) was added dropwise thionyl chloride (10.02–102.0 mmoles). After being heated under reflux for 5 h, the resulting solution was evaporated under reduced pressure. The residual oil was dissolved in dichloromethane (20–200 mL) and cooled in an ice bath before triethylamine (10.02–102 mmoles) and the desired amine (3.67–37.4) were added. The ice bath was removed and the reaction

mixture was allowed to stir overnight at room temperature or until a reasonable conversion was reached (TLC control). This solution was then washed with a 10% K_2CO_3 aqueous solution. The organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure to give the crude yellow solid **8c** or an oily residue. In the latter case, the residue was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with $MeOH/CH_2Cl_2$ gradients to give the product as a orange or a white solid. The obtained compounds were further recrystallized from the specified solvent. Experimental data for the compounds are reported below. Yields were in the range 35–80%, depending on the reactivity of the amine.

[4-(Pyridin-4-ylcarbamoyl)benzyl]carbamic Acid Benzyl Ester, **3a**

It was prepared using compound **2a** (2.00 g, 7.01 mmoles) and 4-aminopyridine (0.73 g, 7.71 mmoles): white solid 2.02 g (5.60 mmoles) (80% yield), mp 148–149°C. ^{1}H-NMR (d_6-DMSO) δ 4.20 (d, 2H, $J = 6.44$ Hz, CH_2), 5.10 (s, 2H, CH_2), 7.25–7.38 (m, 5H, H_{ar}), 7.40 (d, 2H, $J = 8.10$ Hz, H_{ar}), 7.80 (d, 2H, $J = 4.70$ Hz, H_{ar}), 7.90–8.00 (m, 3H, $H_{ar;}$ OCONH), 8.50 (d, 2H, $J = 4.70$ Hz, H_{ar}), 10.50 (signal, 1H, CONH).

{1-[4-(Phenylcarbamoyl)phenyl]ethyl}carbamic Acid Benzyl Ester, **3b**

It was prepared using compound **2b** (1.15 g, 3.84 mmoles) and aniline (0.38 mL, 4.22 mmoles): white crystals 0.64 g (1.72 mmoles) (45% yield), mp 182–183°C (MeOH). ^{1}H-NMR (d_6-DMSO) δ 1.30 (d, 3H, $J = 6.74$ Hz, CH_3), 4.80 (m, 1H, CH), 5.00 (s, 2H, CH_2), 7.10 (m, 1H, H_{ar}), 7.25–7.40 (m, 7H, H_{ar}), 7.45 (d, 2H, $J = 8.21$ Hz, H_{ar}), 7.75 (d, 2H, $J = 7.57$ Hz, H_{ar}), 7.90 (d, 2H, $J = 8.21$ Hz, H_{ar}), 7.95 (d, 1H, $J = 8.00$ Hz, OCONH), 10.20 (signal, 1H, CONH).

{1-[4-(Pyridin-3-ylcarbamoyl)phenyl]ethyl}carbamic Acid Benzyl Ester, **3c**

It was prepared using compound **2b** (1.00 g, 3.34 mmoles) and 3-aminopyridine (0.35 g, 3.67 mmoles): white solid 0.50 g (1.33 mmoles) (40% yield), mp 164–165°C. ^{1}H-NMR (d_6 DMSO) δ 1.30 (d, 3H, $J = 7.00$ Hz, CH_3), 4.75 (m, 1H, CH), 5.00 (s, 2H, CH_2), 7.20–7.45 (m, 6H, H_{ar}), 7.48 (d, 2H, $J = 7.45$ Hz, H_{ar}), 7.80–8.10 (m, 3H, H_{ar}; OCONH), 8.20 (d, 1H, $J = 7.92$ Hz, H_{ar}), 8.35 (m, 1H, H_{ar}), 9.00 (s, 1H, H_{ar}), 10.40 (signal, 1H, CONH).

{1-[4-(1-Benzyl-piperidin-4-ylcarbamoyl)phenyl]ethyl}carbamic Acid Benzyl Ester, **3d**

It was prepared using compound **2b** (1.00 g, 3.34 mmoles) and 4-amino-1-benzylpiperidine (0.64 g, 3.67 mmoles): white crystals 1.10 g (2.34 mmoles) (70% yield), mp 169–170°C (toluene). ^{1}H-NMR ($CDCl_3$) δ 1.45 (d, 3H, $J = 6.90$ Hz, CH_3), 1.55 (m, 2H, CH_2 or 2CH), 2.00 (m, 2H, CH_2 or 2CH), 2.20 (m, 2H, CH_2 or 2CH), 2.85 (m, 2H, CH_2 or 2CH), 3.55 (s, 2H, CH), 4.00 (m, 1H, CH), 4.85 (m, 1H, CH), 5.10 (s, 1H, CH), 6.00 (d, 1H, $J = 8.16$ Hz, OCONH), 7.20–7.45 (m, 13H, $H_{ar;}$ CONH), 7.60 (d, 2H, $J = 8.16$ Hz, H_{ar}).

4-Acetyl-*N*-(4-cyanophenyl)benzamide, **8a**

It was prepared using 4-acetylbenzoic acid **6a** (3.00 g, 18.27 mmoles) and 4-aminobenzonitrile **7a** (2.37 g, 20.09 mmoles): white crystals 1.69 g (6.40 mmoles) (35% yield), mp 199–200°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 2.65 (s, 3H, CH_3), 7.85 (d, 2H, $J = 8.65$ Hz, H_{ar}), 8.00 (d, 2H, $J = 8.65$ Hz, H_{ar}), 8.05–8.14 (m, 4H, H_{ar}), 10.80 (signal, 1H, CONH).

4-Acetyl-*N*-methyl-*N*-pyridin-4-ylbenzamide, **8b**

It was prepared using compound **6a** (1.50 g, 9.13 mmoles) and methyl-pyridin-4-ylamine **7b** (1.09 g, 10.05 mmoles): orange crystals 0.86 g (3.38 mmoles) (37% yield), mp 137–138°C (cyclohexane/toluene = 3/7). ^{1}H-NMR (d_6-DMSO) δ 2.55 (s, 3H, CH_3), 3.42 (s, 3H, CH_3), 7.20 (d, 2H, $J = 6.28$ Hz, H_{ar}), 7.45 (d, 2H, $J = 8.08$ Hz, H_{ar}), 7.85 (d, 2H, $J = 8.08$ Hz, H_{ar}), 8.40 (d, 2H, $J = 6.28$ Hz, H_{ar}).

N-(4-acetylphenyl)isonicotinamide, **8c**

It was prepared using isonicotinic acid **6b** (3.00 g, 24.36 mmoles) and 4-aminoacetophenone **7c** (3.62 g, 26.80 mmoles): yellow crystals 3.98 g (16.56 mmoles) (68% yield), mp 188–189°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 2.57 (s, 3H, CH_3), 7.89 (d, 2H, $J = 4.76$ Hz, H_{ar}), 7.94 (d, 2H, $J = 8.44$ Hz, H_{ar}), 8.01 (d, 2H, $J = 8.44$ Hz, H_{ar}), 8.82 (d, 2H, $J = 4.76$ Hz, H_{ar}), 10.80 (signal, 1H, CONH).

4-Cyano-*N*-pyridin-4-ylbenzamide, **13**

It was prepared using 4-cyanobenzoic acid **12** (5.00 g, 34.0 mmoles) and 4-aminopyridine (3.52 g, 37.4 mmoles): white crystals 3.94 g (17.68 mmoles) (52% yield), mp 190–191°C (MeOH). ^{1}H-NMR (d_6-DMSO) δ 7.80 (d, 2H, $J = 4.96$ Hz, H_{ar}), 8.05 (d, 2H, $J = 6.52$ Hz, H_{ar}), 8.15 (d, 2H, $J = 6.52$ Hz, H_{ar}), 8.55 (d, 2H, $J = 4.96$ Hz, H_{ar}), 10.90 (signal, 1H, CONH).

General Procedure for the Preparation of Compounds 4a–d

To a stirred solution of the appropriated amide **3a–d** (0.66–2.80 mmoles) in methanol (25–100 mL) was added 10% palladium on carbon (0.037–0.15 g), and the mixture was hydrogenated for 2–4 h at room temperature and under atmospheric pressure until TLC showed a reasonable conversion. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure to give a colorless oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm). Elution with

$MeOH/CH_2Cl_2$ gradients gave a solid or a colorless oil. In this last case, the residue was transformed into its hydrochloride salt and recrystallized from the specified solvent. Experimental data for the compounds are reported below.

4-Aminomethyl-*N*-pyridin-4-ylbenzamide, Dihydrochloride, **4a**

It was prepared using compound **3a** (1.00 g, 2.80 mmoles): white crystals 0.50 g (1.68 mmole) (60% yield), mp >260°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 4.20 (m, 2H, CH_2), 7.70 (d, 2H, $J = 8.30$ Hz, H_{ar}), 8.15 (d, 2H, $J = 8.30$ Hz, H_{ar}), 8.45 (d, 2H, $J = 5.90$ Hz, H_{ar}), 8.65 (signal, 3H, NH_3^+), 8.75 (d, 2H, $J = 5.90$ Hz, H_{ar}), 12.00 (signal, 1H, CONH), 15.50 (signal, 1H, NH^+). Found: C, 51.89; H, 5.03; N, 13.90. $C_{13}H_{13}N_3O.2HCl$ requires: C, 51.96; N, 5.00; N, 14.00%.

4-(1-Aminoethyl)-*N*-phenylbenzamide, **4b**

It was prepared using compound **3b** (0.70 g, 1.80 mmole): white crystals 0.38 g (1.59 mmoles) (85% yield), mp = 126–127°C (toluene). ^{1}H-NMR (d_6-DMSO) δ 1.25 (d, 3H, $J = 6.93$ Hz, CH_3), 2.10 (signal, 2H, NH_2), 4.10 (m, 1H, CH), 7.10 (m, 1H, H_{ar}), 7.35 (m, 2H, H_{ar}), 7.50 (d, 2H, $J = 8.22$ Hz, H_{ar}), 7.75 (d, 2H, $J = 7.79$ Hz, H_{ar}), 7.90 (d, 2H, $J = 8.22$ Hz, H_{ar}), 10.20 (signal, 1H, CONH). Found: C, 75.01; H, 6.55; N, 11.80. $C_{15}H_{16}N_2O$ requires: C, 74.98; N, 6.71; N, 11.66%.

4-(1-Aminoethyl)-*N*-pyridin-3-ylbenzamide, Dihydrochloride, **4c**

It was prepared using compound **3c** (0.25 g, 0.66 mmole): white crystals 0.16 g (0.50 mmole) (75% yield), mp > 260°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 1.50 (d, 3H, $J = 6.72$ Hz, CH_3), 4.50 (m, 1H, CH), 7.70 (d, 2H, $J = 8.28$ Hz, H_{ar}), 8.00 (m, 1H, H_{ar}), 8.15 (d, 2H, $J = 8.28$ Hz, H_{ar}), 8.60 (d, 1H, $J = 5.17$ Hz, H_{ar}), 8.70 (signal, 3H, NH_3^+), 8.82 (d, 1H, $J = 8.27$ Hz, H_{ar}), 9.40 (s, 1H, H_{ar}), 11.50 (signal, 1H, CONH). Found: C, 53.41; H, 5.49; N, 13.25. $C_{14}H_{15}N_3O.2HCl$ requires: C, 53.51; N, 5.45; N, 13.37%.

4-(1-Aminoethyl)-*N*-(1-benzylpiperidin-4-yl)benzamide, **4d**

It was prepared using compound **3d** (0.80 g, 1.70 mmole): white solid 0.47 g (1.40 mmole) (82% yield), mp = 128–129°C. ^{1}H-NMR (d_6-DMSO) δ 1.00 (d, 3H, $J = 5.70$ Hz, CH_3), 1.55 (m, 2H, CH_2), 1.80 (m, 2H, CH_2), 1.90–2.30 (m, 3H, CH_2; NH_2), 2.80 (m, 2H, CH_2), 3.45 (s, 2H, CH_2), 3.75 (m, 1H, CH), 4.00 (m, 1H, CH), 7.10–7.35 (m, 5H, H_{ar}), 7.45 (d, 2H, $J = 9.55$ Hz, H_{ar}), 7.75 (d, 2H, $J = 9.55$ Hz, H_{ar}), 8.10 (d, 1H, $J = 8.79$ Hz, CONH).

4-(1-Aminoethyl)-*N*-piperidin-4-ylbenzamide, Dihydrochloride, Monohydrate, **5**

To a stirred solution of 4-(1-aminoethyl)-*N*-(1-benzylpiperidin-4-yl)benzamide **4d** (0.50 g, 1.5 mmole) in 4.4% formic acid–methanol (40 mL) was added black palladium (0.50 g), and the mixture was placed under a nitrogen atmosphere for 6 h at room temperature. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure to give a colorless oily residue which was dissolved in a minimum volume of a 10% K_2CO_3 aqueous solution. The organic layer was extracted with 1-butanol and evaporated under reduced pressure to give a crude oily residue which was transformed into its hydrochloride salt and recrystallized from EtOH to give a white crystalline solid (0.20 g, 0.60 mmole) (40% yield) **5**, mp > 260°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 1.50 (d, 3H, $J = 6.90$ Hz, CH_3), 1.80 (m, 2H, CH_2), 1.95 (m, 2H, CH_2), 3.00 (m, 2H, CH_2), 3.30 (m, 2H, CH_2), 4.00 (m, 1H, CH), 4.45 (m, 1H, CH), 7.55 (d, 2H, $J = 8.11$ Hz, H_{ar}), 7.95 (d, 2H, $J = 8.11$ Hz, H_{ar}), 8.50 (d, 1H, $J = 7.38$ Hz, CONH), 8.90 (signal, 5H, NH_2^+ and NH_3^+). Found: C, 50.00; H, 7.17; N, 12.53. $C_{14}H_{21}N_3O.2HCl$, H_2O requires: C, 49.66; N, 7.44; N, 12.41%.

General Procedure for the Preparation of Compounds 9a–c

To a solution of the appropriate amide (**8a–c**) (3.93–7.07 mmoles) in methanol (30–50 mL) was added pyridine (1.42–2.56 mL, 17.68–31.81 mmoles) and a solution of hydroxylamine hydrochloride (1.09–1.96 g, 15.7–28.28 mmoles) in water (4–7 mL). The reaction was stirred under reflux for 2–5 h until TLC showed a reasonable conversion.

After cooling, the precipitate was filtered off and washed with absolute EtOH to give the crude white product **9c** or the reaction was concentrated under reduced pressure to give an oily residue. In this latter case, the residue was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with $MeOH/CH_2Cl_2$ gradients to give a yellow or a white solid. The obtained compounds were further recrystallized from the specified solvent. Experimental data for the compounds are reported below.

N-(4-cyanophenyl)-4-(1-hydroxyiminoethyl)benzamide, **9a**

It was prepared using compound **8a** (1.10 g, 4.16 mmoles): yellow solid 0.69 g (2.48 mmoles) (60% yield), mp = 241–242°C. ^{1}H-NMR (d_6-DMSO) δ 2.15 (s, 3H, CH_3), 7.75–7.90 (m, 4H, H_{ar}), 7.95–8.10 (m, 4H, H_{ar}), 10.70 (signal, 1H, CONH), 11.50 (signal, 1H, OH).

4-(1-Hydroxyiminoethyl)-*N*-methyl-*N*-pyridin-4-ylbenzamide, **9b**

It was prepared using compound **8b** (1.00 g, 3.93 mmoles): white crystals 0.55 g (2.04 mmoles) (55% yield), mp = 125–126°C (toluene). ^{1}H-NMR

(d_6-DMSO) δ 2.10 (s, 3H, CH_3), 3.42 (s, 3H, CH_3), 7.20 (d, 2H, $J = 6.15$ Hz, H_{ar}), 7.34 (d, 2H, $J = 8.45$ Hz, H_{ar}), 7.58 (d, 2H, $J = 8.45$ Hz, H_{ar}), 8.40 (d, 2H, $J = 6.15$ Hz, H_{ar}), 11.40 (signal, 1H, OH).

N-[4-(1-hydroxyiminoethyl)-phenyl]isonicotinamide, **9c**

It was prepared using compound **8c** (1.70 g, 7.07 mmoles): white crystals 1.00 g (3.89 mmoles) (55% yield), mp $>$ 260°C (EtOH/DMF = 3:7). ^{1}H-NMR (d_6-DMSO) δ 2.15 (s, 3H, CH_3), 7.67 (d, 2H, $J = 8.91$ Hz, H_{ar}), 7.81 (d, 2H, $J = 8.91$ Hz, H_{ar}), 7.87 (d, 2H, $J = 6.20$ Hz, H_{ar}), 8.80 (d, 2H, $J = 6.20$ Hz, H_{ar}), 10.55 (signal, 1H, CONH), 11.20 (signal, 1H, OH).

General Procedure for the Preparation of Compounds 10a–c

To a solution of the appropriate oxime (**9a–c**) (1.67–2.14 mmoles) in ethanol (30–35 mL) was added concentrated ammonium hydroxide (7.5–9.0 mL) and Raney nickel (0.055–0.070 g). The reaction mixture was stirred under a pressure of hydrogen (approximatively 7 atmospheres), at room temperature, for 4–16 h. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure to give a colorless oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with $MeOH/CH_2Cl_2$ gradients to give a white solid **10c** or a colored oil. In this last case, the residue could be transformed into its hydrochloride salt without further purification. Experimental data for the compounds are reported below.

4-(1-Aminoethyl)-*N*-(4-aminomethyl-phenyl)-benzamide, Dihydrochloride, **10a**

It was prepared using compound **9a** (0.60 g, 2.14 mmoles): white solid 0.22 g (0.64 mmoles) (30% yield), mp $>$ 260°C. ^{1}H-NMR (d_6-DMSO) δ 1.50 (d, 3H, $J = 6.58$ Hz, CH_3), 4.00 (s, 2H, CH_2), 4.50 (m, 1H, CH), 7.45 (d, 2H, $J = 8.34$ Hz, H_{ar}), 7.65 (d, 2H, $J = 7.90$ Hz, H_{ar}), 7.85 (d, 2H, $J = 8.34$ Hz, H_{ar}), 8.05 (d, 2H, $J = 7.90$ Hz, H_{ar}), 8.60 (signal, 6H, NH_3^+), 10.50 (signal, 1H, CONH). Found: C, 55.85; H, 6.26; N, 11.87. $C_{16}H_{19}N_3O.2HCl$ requires: C, 56.14; N, 6.18; N, 12.27%.

4-(1-Aminoethyl)-*N*-methyl-N-pyridin-4-ylbenzamide, **10b**

It was prepared using compound **9b** (0.45 g, 1.67 mmole): yellow oil 0.27 g (1.08 mmole) (65% yield). ^{1}H-NMR ($CDCl_3$) δ 1.30 (d, 3H, $J = 6.24$ Hz, CH_3), 1.70 (signal, 2H, NH_2), 3.50 (s, 3H, CH_3), 4.10 (m, 1H, CH), 6.95 (d, 2H, $J = 6.93$ Hz, H_{ar}), 7.22 (d, 2H, $J = 8.16$ Hz, H_{ar}), 7.30 (d, 2H, $J = 8.16$ Hz, H_{ar}), 8.42 (d, 2H, $J = 6.93$ Hz, H_{ar}). Found: C, 70.35; H, 6.47; N, 16.36. $C_{15}H_{17}N_3O$ requires: C, 70.50; N, 6.66; N, 16.45%.

N-[4-(1-aminoethyl)-phenyl]isonicotinamide, **10c**

It was prepared using compound **9c** (0.50 g, 1.95 mmole): white crytals 0.27 g (1.11 mmole) (57% yield), mp = 159–160°C (EtOAc). ^{1}H-NMR (d_6-DMSO) δ 1.20 (d, 3H, $J = 6.69$ Hz, CH_3), 1.80 (signal, 2H, NH_2), 4.00 (m, 1H, CH), 7.35 (d, 2H, $J = 8.37$ Hz, H_{ar}), 7.65 (d, 2H, $J = 8.37$ Hz, H_{ar}), 7.85 (d, 2H, $J = 6.06$ Hz, H_{ar}), 8.75 (d, 2H, $J = 6.06$ Hz, H_{ar}), 10.50 (signal, 1H, CONH).

4-(1-Aminoethyl)-N-(4-cyanophenyl)benzamide, 11

To a solution of 4-acetyl-*N*-(4-cyanophenyl)benzamide **8a** (0.90 g, 3.5 mmoles) and 3 Å molecular sieves (2 g) in anhydrous methanol (30 mL) was added sodium cyanoborohydride (0.15 g, 2.5 mmoles) and ammonium acetate (2.60 g, 35.0 mmoles). The mixture was placed under a nitrogen atmosphere for 3 days at room temperature. The molecular sieves were filtered off, and the filtrate was concentrated under reduced pressure to give an oily residue which was purified by column chromatography using silica gel 60 as stationary phase (Merck silica gel 60, 70–230 mesh, 63–200 μm) and eluted with $MeOH/CH_2Cl_2$ gradients to give a white solid (0.09 g, 0.34 mmole) (10% yield) **11**, mp = 167–168°C. ^{1}H-NMR (d_6-DMSO) δ 1.25 (d, 3H, $J = 6.73$ Hz, CH_3), 3.30 (signal, 2H, NH_2), 4.10 (m, 1H, CH), 7.56 (d, 2H, $J = 8.53$ Hz, H_{ar}), 7.82 (d, 2H, $J = 8.98$ Hz, H_{ar}), 7.92 (d, 2H, $J = 8.53$ Hz, H_{ar}), 8.02 (d, 2H, $J = 8.98$ Hz, H_{ar}), 10.50 (signal, 1H, CONH).

4-Carbamimidoyl-N-pyridin-4-yl-benzamide, Hydrochloride, 14

To a solution of 4-cyano-*N*-pyridin-4-ylbenzamide **13** (1.00 g, 4.5 mmoles) in dry chloroform (40 mL) was added methanol (4 mL). The solution was cooled to 0°C and a vigorous stream of dry hydrogen chloride introduced. A white precipitate formed after 30 min. The mixture was kept at room temperature for 24 h under a low pressure of hydrogen chloride. The white solid was filtered, washed with chloroform, and shaken with dry methanol (20 mL) over a stream of dry ammonia. Most of the solid dissolved. The mixture was kept 24 h at room temperature, filtered, evaporated to a small volume and chilled. The crude white solid residue was filtered and recrystallized from EtOH to give a white crystalline solid (0.19 g, 0.67 mmole) (15% yield) **14**, mp $>$ 260°C (EtOH). ^{1}H-NMR (d_6-DMSO) δ 7.85 (d, 2H, $J = 6.40$ Hz, H_{ar}), 8.00 (d, 2H, $J = 8.34$ Hz, H_{ar}), 8.20 (d, 2H, $J = 8.34$ Hz, H_{ar}),

8.50 (d, 2H, $J = 6.40$ Hz, H_{ar}), 9.50 (signal, 3H, NH_3^+), 11.00 (signal, 1H, CONH). Found: C, 56.55; H, 4.46; N, 20.29. $C_{13}H_{12}N_4O{\cdot}HCl$ requires: C, 56.37; N, 4.69; N, 20.23%.

Pharmacology

Chemicals and Drugs

Texas Red-Dnase I was obtained from Molecular Probe (Leiden, The Netherland). All other reagents were purchased from Sigma (Saint Quentin Fallavier, France).

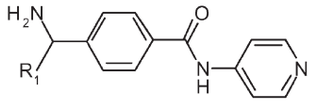
Isometric Tension Measurement in Skinned Fibers

Guinea-pigs were stunned and then killed by cervical dislocation. The mesenteric artery was collected in physiological saline solution (PSS, in mM; 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 10 Tris, pH 7.4 with HCl) cleaned of fat and adherent connective tissue and longitudinally opened. The endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. Small muscle strips (approximately 200 μm wide and 4 mm long) were isolated from the media and tied at each end with a single silk thread to the tips of two needles, one of which was connected to a force transducer (AE 801, SensoNor, Horten, Norway). Strips were placed in a well on a bubble plate filled with PSS and stretched to about 1.3 resting length. The solution was rapidly changed by sliding the plate to an adjacent well. After measuring contraction evoked by high-K^+ solution, the strips were incubated in the normal relaxing solution (in mM: 85 KCl, 5 $MgCl_2$, 5 Na_2ATP, 5 creatine phosphate, 2 EGTA and 20 Tris-maleate, brought to pH 7.1 at 25°C with KOH) for a few minutes, followed by treatment with β-escin (50–70 μM) in the relaxing solution for 35 min at 25°C as previously described.[26] The skinned muscle strip was then washed several times with fresh relaxing solution containing 10 mM EGTA. Calmodulin (1.5 μM) was added to the bathing solutions throughout the experiments. Tension developed by permeabilized muscle strips was measured in activating solutions, containing 10 mM EGTA and a specified amount of $CaCl_2$ to give a desired concentration of free Ca^{2+}.[26] Ca^{2+} sensitization was induced at in low-Ca^{2+} solution ($-\log[Ca^{2+}] = 6.3$; $pCa = 6.3$) by adding GTPγS (10 μM) to stimulate G-proteins. When tension reached a steady state, the inhibitory action of compounds was tested by adding increasing concentrations. The amplitude of tension was measured at each concentrations, then expressed as a percentage of the maximal control response recorded before the addition of the inhibitor. Activity of each compound was quantified by its IC_{50}, defined as the concentration of compound giving 50% of its maximal inhibitory effect (Table I—Test A-1) (Figure 2).

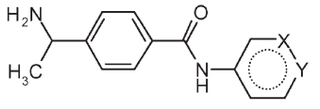
Smooth Muscle Cell Culture

Smooth muscle cells from young rat (45 g) aorta were isolated by enzymatic dissociation as previously described.[27] Cells were cultured in DMEM with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Secondary cultures were obtained by serial passages after the cells were harvested with 0.5 g/l trypsin and 0.2 g/l EDTA (Trypsin-EDTA) and reseeded in fresh DMEM containing 10% FCS and antibiotics.

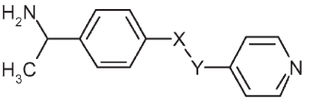
TABLE I Inhibition values of the 10^{-5} M GTPγS-induced contraction (Test A-1) and of the stress fiber formation (Test A-2)



Y-32885 ; 4a ; 14



4b,c ; 5 ; 10a ; 11



10b,c

No.	R_1	X	Y	Test A-1 IC_{50} (μM)	Test A-2% of inhibition at 10^{-5} M
Y-32885 (racemic)	H_3C			0.8	75.1 ± 3.3
Y-32885 (S)	H_3C			1.0	74.2 ± 1.5
Y-32885 (R)	H_3C			0.2	84.7 ± 2.1
4a	H			30	31.9 ± 3.9
4b		CH	CH	inactive	inactive
4c		N	CH	inactive	inactive
5		CH_2	NH	inactive	inactive
10a		CH	$C–CH_2–NH_2$	inactive	inactive
10b		C = O	$N–CH_3$	inactive	inactive
10c		NH	C = O	inactive	23 ± 5.4
11		CH	C–CN	inactive	inactive
14	HN			5	inactive

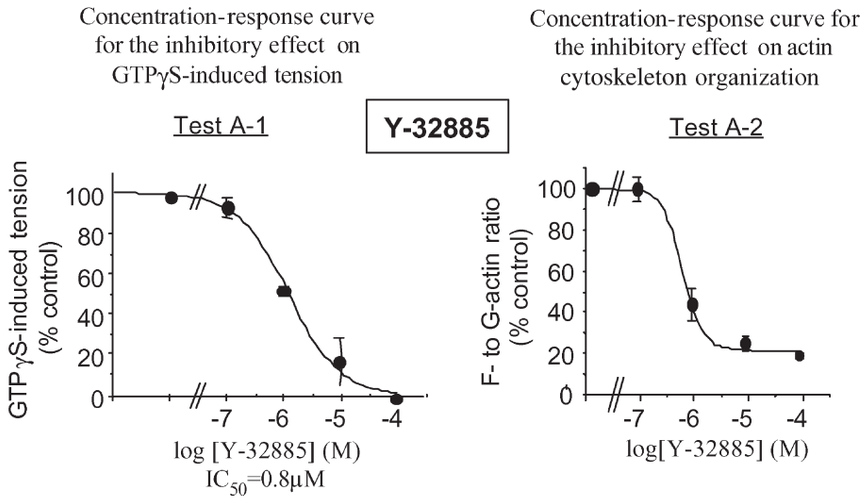


FIGURE 2 Inhibitory action of Y-32885 (racemic) in the two tests.

Actin Staining

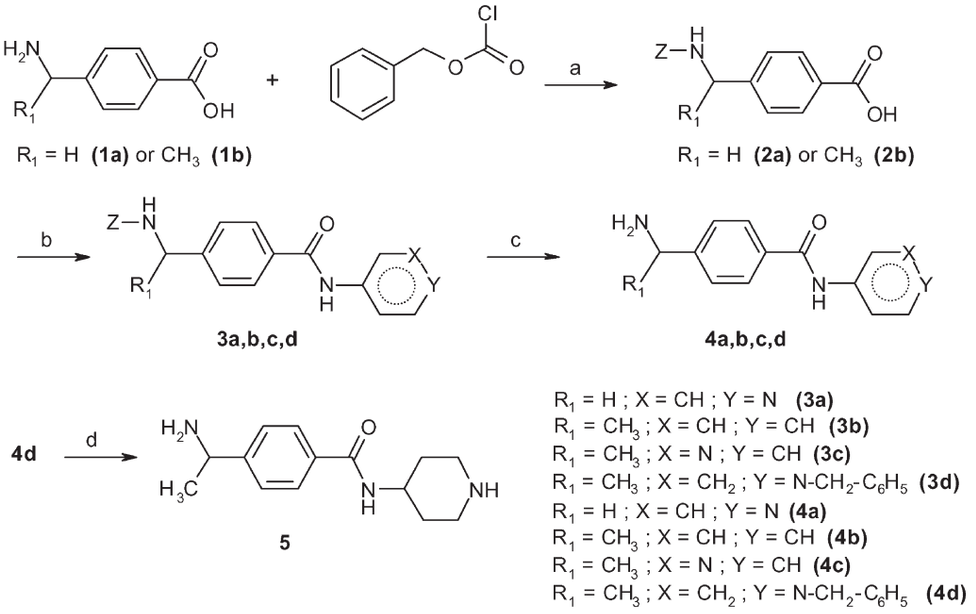
After dissociation, aortic myocytes were cultured in DMEM with 10% FCS on glass coverslips for 2 days. The cells were maintained in culture for another day (24 h) in the absence or presence of the molecules to be tested. Cells were then fixed for 30 min in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and then rinsed in phosphate-buffered saline (PBS). For polymerized (F) actin staining, cells were incubated with FITC-conjugated phalloidin (5 μg/ml) for 45 min at room temperature then washed with PBS. Actin staining was also performed with a monoclonal anti-α-smooth muscle actin antibody followed by FITC-conjugated anti-mouse antibody which gave results similar to those obtained with FITC-conjugated phalloidin. When dual labeling was performed, cells were simultaneously stained with FITC-conjugated phalloidin and Texas red-labeled DNase I (10 μg/ml) to localize monomeric G-actin,[28] and then washed in PBS. Coverslips were mounted on a glass slide and examined with a fluorescence microscope (Eclipse E-600, Nikon, Champigny-sur-Marne, France). The background fluorescence signal was estimated by collecting planes from areas of the slide without cells and was electronically subtracted before analysis. Images were collected with a cool-SNAP camera (Princeton Instruments, Evry, France) and stored and analyzed using Metamorph software (Universal Imaging, West Chester, PA). For each area examined, images of FITC-phalloidin and Texas Red-DNase I fluorescence were collected. The time of measurements and image capturing and the image intensity gain at both wavelengths were optimally adjusted and kept constant. The ratio of fluorescence of FITC-phalloidin and Texas Red-DNase I (F- to G-actin ratio), used to quantify actin cytoskeleton organization, and so stress fiber formation, was calculated for at least 20 cells in each experimental condition and expressed as a percentage of the ratio obtained under control condition. A decrease in the F- to G-actin ratio was assumed to represent depolymerization of actin filaments and so the percentage of inhibition of the compounds, expressed at a 10^{-5} M concentration (Table I—Test A-2) (Figure 2).

RESULTS AND DISCUSSION

Chemistry

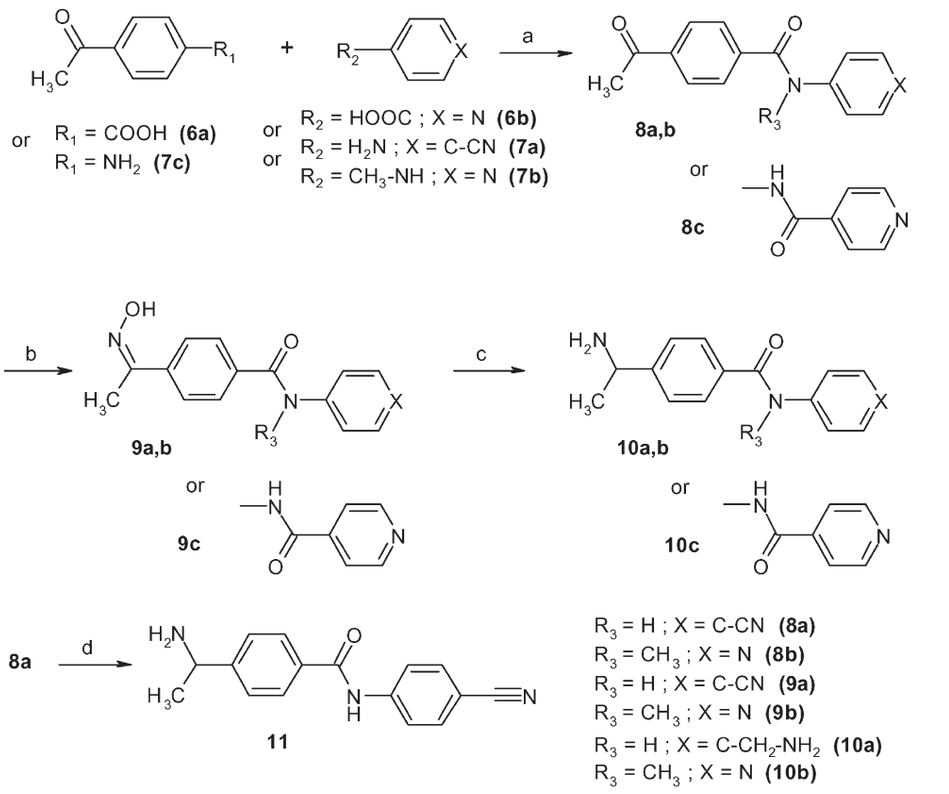
Y-32885 was prepared according to existing literature procedure[24,29] and the separation of the enantiomers was described by Les Laboratoires Servier. The acids and amines used as starting materials were commercially available, except acids **1b**, **6a** and amine **7b** which were prepared according to previously described methods.[30,31]

Amines **4a**–**d** were obtained following the synthetic route described in Scheme 1.[24,29] Condensation of aminoacids **1a**–**b** with benzyl chloroformate in a dioxane/water mixture under basic conditions gave the *N*-Z-protected acids **2a**–**b**. These compounds were first treated with thionyl chloride in methylene chloride and the unisolated corresponding acid chlorides were condensed with the appropriate amines to afford amides **3a**–**d** which led to the desired amines **4a**–**d**, by hydrogenolysis in the presence of 10% palladium on carbon in methanol under an atmospheric hydrogen pressure. The removal of the *N*-benzyl protecting group from **4d**, by catalytic transfer hydrogenation with 4.4% formic acid in methanol and black palladium, gave the piperidine derivative **5**.[32]

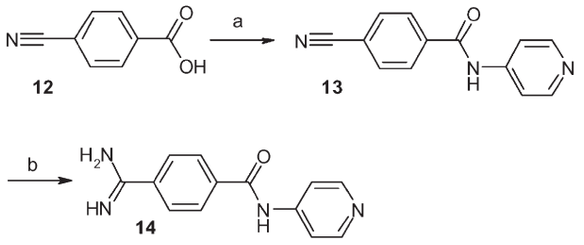


SCHEME 1 Synthesis of compounds **4a**–**d** and **5**. Reagents: (a) NaOH 1N, dioxane; (b) i) $SOCl_2$, CH_2Cl_2 ii) 4-aminopyridine or aniline or 3-aminopyridine or 4-amino-1-benzylpiperidine, CH_2Cl_2; (c) H_2, 10% Pd/C, MeOH; (d) 4.4% HCOOH/MeOH, black palladium.

The synthesis of amines **10a**–**c** and **11** is shown in Scheme 2. Treatment of the acids **6a**–**b** with thionyl chloride in methylene chloride and further with the appropriate amines afforded the amides **8a**–**c**. The use of hydroxylamine hydrochloride and pyridine in a water/methanol mixture led to oximes **9a**–**c** which gave the desired amines **10a**–**c**, by hydrogenolysis in the presence of Raney nickel in ethanol and



SCHEME 2 Synthesis of compounds **10a**–**c** and **11**. Reagents: (a) $SOCl_2$, CH_2Cl_2; (b) $NH_2OH{\cdot}HCl$, pyridine, H_2O/MeOH; (c) H_2, 7 atm/Raney-Ni, EtOH/NH_4OH; (d) $NaBH_3CN$, $CH_3CO_2NH_4$, MeOH.



SCHEME 3 Synthesis of the amidine **14**. Reagents: (a) i) $SOCl_2$, CH_2Cl_2 ii) 4-aminopyridine, CH_2Cl_2; (b) i) HCl, MeOH, $HCCl_3$ ii) NH_3, MeOH.

concentrated ammonium hydroxide under a 7 atmospheres hydrogen pressure. Amine **11** was obtained from **8a** by reductive amination with sodium cyanoborohydride and ammonium acetate in methanol according to a previously described procedure.[33,34]

Amidine **14** was synthesized by the route illustrated in Scheme 3, from 4-cyanobenzoic acid **12**. Treatment with thionyl chloride and further with 4-aminopyridine gave the amide **13** which was first treated with methanol and dry hydrogen chloride. The corresponding imido-ether hydrochloride was not isolated and condensed with ammonia to give the amidine.[35]

Pharmacology

The aim of this study was to evaluate the role of the structural features of compound Y-32885 with regard to its Rho-kinase inhibitory activity.

We therefore rationally modified the three functional groups which could be implicated in the interaction with the enzyme active site: (i) the 1-aminoethyl chain (ii) the 4-pyridyl heterocycle (iii) the amide spacer:

i) The 1-aminoethyl side chain bears a chiral center which does not seem to be an essential feature, the racemate being nearly as active as the R-(+) enantiomer (Table I). We first decided to suppress the chiral center by replacing the methyl group by hydrogen and prepared compound **4a** which is 40 fold and 2 fold less active than Y-32885 in the two tests A-1 and A-2, respectively. On the other hand, we replaced this methyl by an imino group to obtain the amidine **14** which is only 6 fold less active on the test A-1 but inactive on the test A-2. This discrepancy could be explained by a weak penetration of **14** through the plasmic membrane. Taken together these results seem to imply the role of both lipophilicity and steric parameters at this level of interactions.
ii) The pyridyl nitrogen could be involved in ionic or hydrogen bonding with the enzyme active site. This hypothesis is corroborated by the various pharmacomodulations carried out on this feature: suppression of the nitrogen atom (**4b**), isomerisation to the 3-position (**4c**) and replacement by a cyano (**11**) or an aminomethyl (**10a**) group led to inactive compounds in the two tests. The same results are obtained with the corresponding aliphatic heterocycle (**5**). These modifications indicate that a strict spatial arrangement of this pyridyl nitrogen is required for the interaction and that additional $\pi-\pi$ interactions could be involved at this level.
iii) Generally, an amide group has a structural role in holding other functionalities in a particular geometry and/or is concerned with hydrogen bonds interactions with the drug target. This is probably the case for the amide spacer of compound Y-32885 since methylation of the nitrogen (**10b**) and synthesis of the retroamide (**10c**) induced a loss of the inhibitory activity.

CONCLUSION

Our main objective was to clarify the relationships between the structure of compound Y-32885 and its Rho-kinase inhibitory activity. All the pharmacomodulations achieved on the main structural features of this lead compound result in a decrease or a loss of activity, showing that its interactions with the enzyme active site are particularly definite and sensitive to structural variations. The pharmacophoric pattern constituted by a 4-pyridine heterocycle and a 1-aminoalkyl amine separated by a spacer bearing an amide function, is of utmost importance.

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