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PAPER

A practical synthesis of Rho-Kinase inhibitor Y-27632 and fluoro derivatives and their evaluation in human pluripotent stem cells†

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A practical synthesis of the Rho-Kinase inhibitor Y-27632 and two new fluoro derivatives was achieved in seven steps and with a good overall yield of 45% starting from commercially available (*R*)-1-phenylethylamine. Compared to Y-27632 the new fluoro derivatives showed reduced or no effect on hPSC vitality and expansion after dissociation in human pluripotent stem cells.

Introduction

(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)-cyclohexane carboxamide dihydrochloride (**1**) (Y-27632, Fig. 1) is highly potent, cell-permeable and selective ROCK (Rho-associated coiled coil forming protein serine/threonine kinase) inhibitor. This compound has played a major role for better understanding the physiological roles of this Rho-kinase. Furthermore, Y-27632 (**1**) is widely utilised and tested in the treatment of various diseases. These include different cellular functions, cell adhesion, cell motility, vascular and smooth muscle contraction, cytokinesis,[1a,b] cardiovascular diseases, such as hypertension and arteriosclerosis,[1c] bronchial asthma,[1d] cancer,[1e] Alzheimer's disease,[1f] coronary artery spasm[1c,g] and vasospastic angina.[1c,h] Recently, we and others disclosed the first applications of **1** in human pluripotent embryonic stem (hPS) cell research, particularly in scalable mass expansion in suspension culture which is of high relevance to clinical applications.[2] Due to its broad importance this ROCK inhibitor **1** or derivatives should become pharmaceutical candidates for several future clinical applications.

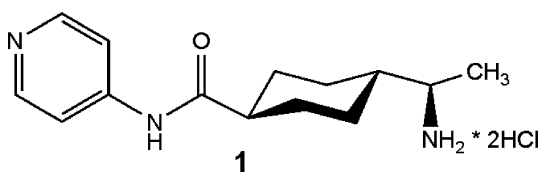

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Fig. 1 Rho-kinase inhibitor Y-27632 (**1**).

The synthesis of **1** and its analogues was first published by Yoshitomi Pharmaceutical Industries.[3] The α-alkylbenzylamines were used as chiral starting material. However, these reports[3] do not provide sufficient analytical data. In fact, only the melting point and the optical rotation $[\alpha]_D$ +4.6 (*c* 1, MeOH) was reported.[3b]

Later two syntheses of ROCK-inhibitor **1** were published that started from 1,4-cyclohexyldimethanol[4a] and from *trans*-1,4-cyclohexanedicarboxylic acid.[4b] Again, these publications lacked sufficient description of analytical data.

As part of our investigations in stem cell research[2] we first required sufficient amounts of **1** as well as of fluoro analogues. During our efforts to follow the patent descriptions[3] we noted major difficulties as well peculiarities when comparing analytical data with those reported.

Results and discussion

The synthesis of Y-27632 (**1**) commenced from the commercially available and inexpensive (*R*)-1-phenylethylamine (**2**) (Scheme 1) which was transformed according to the published procedure[3c] to the benzoic acid **3** in the three-step sequences that included *N*-acylation (95%), Friedel–Crafts acylation (90%) and haloform reaction (91%).

Hydrogenation (H_2, 5% Ru/C, 26% aqueous ammonia) of the aromatic moiety was the key step of the synthesis. It was carried out in an autoclave under different conditions with respect to pressure and temperature. We repeatedly observed that only one isomer of two possible cyclohexane products was formed, the *N*-acetylated acid **4** or/and the deacetylated acid **5**, respectively (Scheme 1). Lower initial hydrogen pressure, lower temperature and shorter reaction time (50 bar, 90 °C, 6 h, then 120 °C, 6 h) led to the acetamidoethyl acid **4**. Still the starting benzoic acid **3** (*ca* 10–20%) was always detectable in the NMR spectra. When the hydrogenation was carried out at higher pressure and temperature (70 bar, 150 °C) for 1–3 days, formation of a mixture of the two *trans*-isomers **4a** and **5a** in varying ratios were observed. In the following, the resulting mixture of **4a** and **5a** was heated in an

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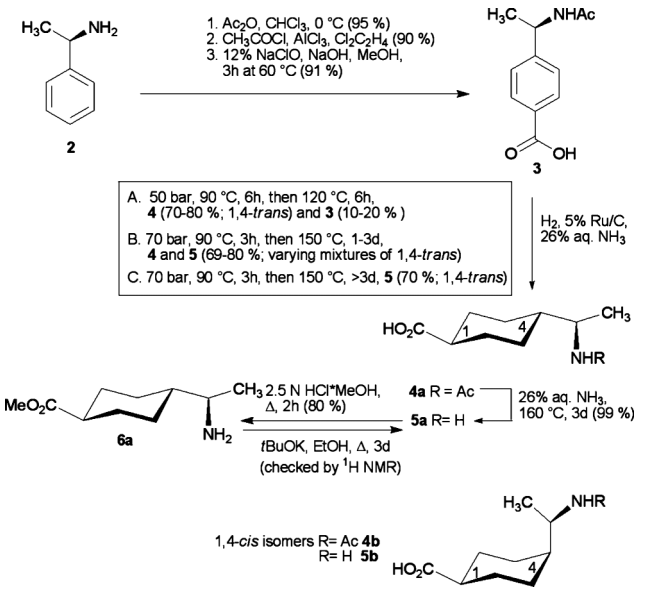


Scheme 1 Synthesis of (*R*)-4-(1-aminoethyl) cyclohexanecarboxylic acid **5a** (1,4-*trans*) and isomerisation attempts.

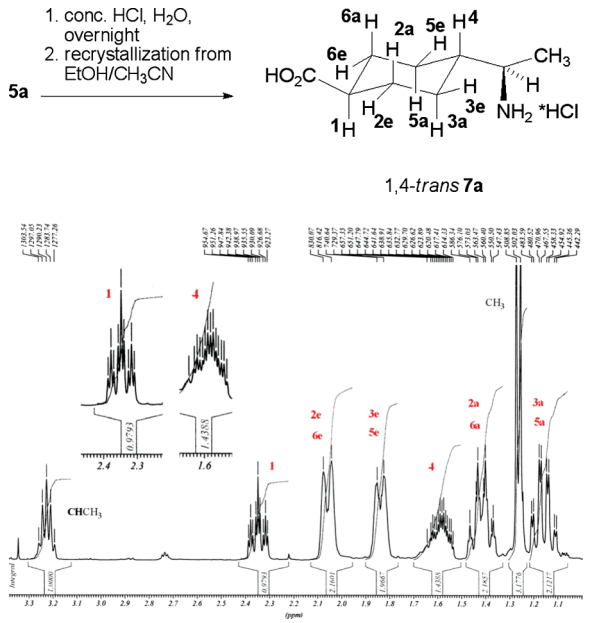
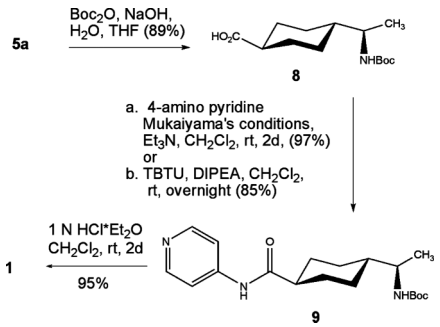


Fig. 2 ^{1}H-NMR spectra of *trans*-(*R*)-4-(1-aminoethyl)cyclohexane carboxylic acid hydrochloride (**7a**).

autoclave with 26% aqueous ammonia at 160 °C for three days which resulted in full conversion to the aminoethyl acid **5a**. Longer reaction times (>3 days) under identical hydrogenation conditions afforded the acid **5a** in one step in 70% yield. In all these hydrogenation experiments the carboxylic acids **4a** or **5a** were isolated as pure 1,4-*trans*-isomers while formation of the 1,4-*cis* isomer **4b** or **5b**, respectively, was not detected. However, comparison of the melting point of our 1,4-*trans*-carboxylic acid **5a** (255 °C) revealed a discrepancy with the reported one (1,4-*trans*-isomer **5a**: mp >290 °C),[5] while the melting point for the 1,4-*cis*-isomer **5b** was reported to be 243 °C. In order to clarify the situation, we more closely analysed the 1,4-configurations in the cyclohexane ring in **4a** and **5a**, respectively. As shown below, two isomerisation experiments on **5a** and **6a**, ^{1}H-NMR analysis of an analytically pure sample of **7a** and final NMR comparison of the synthesised ROCK inhibitor Y-27632 (**1**) with an authentic sample (see ESI†) confirmed the correct stereochemistry.

Firstly, we attempted two isomerisation experiments[3c,5,6] (Scheme 1) on carboxylic acid **5a** and its methyl ester **6a** which was first straightforwardly prepared by esterification[7] using methanolic HCl (1.25 N solution). Thus, both cyclohexane derivatives **5a** and **6a** were heated under refluxing conditions in an ethanolic solution of *t*BuOK for 3 days. NMR-analysis revealed no changes except that ester **6a** was hydrolysed to the carboxylic acid **5a**. From these results it can be concluded that the 1,4-*trans* isomer is the thermodynamically more stable isomer which is also formed under hydrogenation conditions. Recrystallisation of cyclohexanecarboxylic acid hydrochloride **7a** from ethanol-acetonitrile gave an analytically pure sample of **7a** which allowed to assign all coupling constants for the axially oriented protons at H-1 and H-4 ($^3J_{aa}$ = 12 Hz) that further confirmed the desired 1,4-*trans*-configuration (Fig. 2).

Finally, the synthesis of Y-27632 (**1**) was finalised after *N*-Boc protection (89%)[8] and amidation of the carboxylic acid **8** with 4-aminopyridine to yield **9**. For this step two different methods, the Mukaiyama reaction[9] (97%) and the reagent system TBTU/DIPEA[10] (85%) were probed. Finally, removal of the Boc group using 1 N HCl in diethyl ether afforded the target ROCK-inhibitor **1** (Scheme 2).

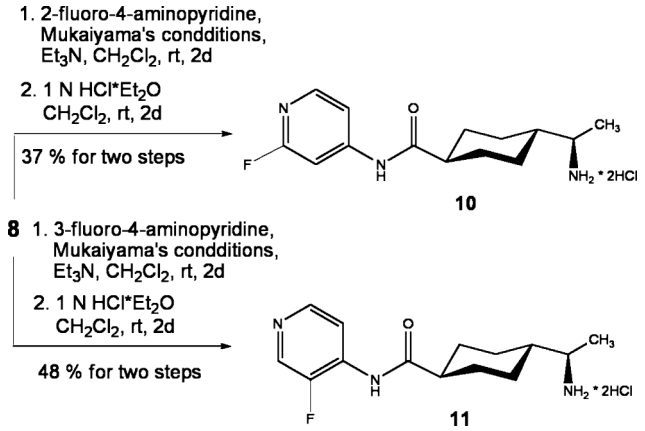


Scheme 2 Synthesis of ROCK-inhibitor Y-27632 (**1**).

In addition, we found that the optical rotations reported in the literature substantially differed from the one that we measured. We measured a specific optical rotation value of ($[\alpha]_D$ −11.5 (*c* 0.5, MeOH) compared to $[\alpha]_D$ +4.6 (*c* 1, MeOH).[3b] Nevertheless, all physical and analytical data were in full agreement with those measured for an authentic sample of **1** (see ESI†).[11] Correctness of our material was proven in biological tests with human embryonic stem (hES) cells. Both the synthetic as well as an authentic sample of Y-27632 showed identical activity.[2]

In order to study the influence of electronic effects in the pyridine ring on ROCK inhibition without introducing large substituents we prepared two new fluoro derivatives **10** and **11** by following the sequence described in Scheme 2 (Scheme 3).

Human pluripotent stem cells (hPSC)—including human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC)—unlike mouse ones, are vulnerable to apoptosis upon dissociation.[2] Based on our fundamental work showing that **1** markedly supports expansion of hPS cells as floating aggregates in a serum-free suspension culture,[2d] we further evaluated the



Scheme 3 Synthesis of fluoro derivatives **10** and **11** of ROCK-inhibitor **1**.

activity of the novel fluoro derivatives. The data presented in Fig. 3 suggest that fluoro derivative **11** at least partially rescued hPSC. This was in the range of 25–40% compared to the activity of **1** regarding the efficiency of supporting cell expansion (Fig. 3 A). In contrast, the activity of fluoro derivative **10** was equivalent to non-supplemented medium controls in the same assay, meaning that survival, re-aggregation and expansion of single-cell dissociated hPSC was not supported (Fig. 3 A-B).

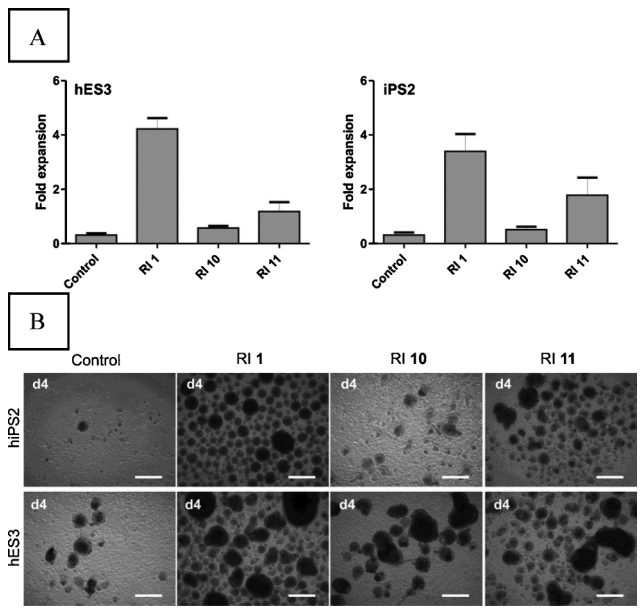


Fig. 3 *In vitro* assessment of **1**, **10** and **11** regarding their efficiency to support survival, re-aggregation and expansion of single cell-inoculated suspension cultures of human ES and human iPS cells. (A) Fold expansion of hESC or hiPSC 4 days after inoculation relative to the seeding density. Controls were cultured in mTeSR medium only. In test cultures mTeSR was supplemented with the respective compound at a 10 μM concentration each. Every bar represents $n = 9$. (B) Representative pictures of aggregates formed in suspension culture 4 days after single cell inoculation in controls or in the presence of the respective compound; scale bars represents 200 μm.

The serine/threonine kinase ROCK is an effector of Rho-dependent signalling and is involved in actin-cytoskeleton assembly, cell motility and contraction. The ROCK protein consists of several domains, an N-terminal region, a kinase catalytic domain, a coiled-coil domain containing a RhoA binding site, and a pleckstrin homology domain. The C-terminal region of ROCK binds to and inhibits the kinase catalytic domains, and this inhibition is reversed by binding RhoA, a small GTPase.

Jacobs and coworkers[12] determined crystal structures of four ROCK protein–ligand complexes containing the inhibitors Y-27632, fasudil (HA-1077), hydroxyfasudil (HA-1100), and a dimethylated analog of fasudil (H-1152P). Y-27632 is a pyridine compound that is chemically distinct from the other three isoquinoline-based ligands. Each of these compounds binds with reduced affinity to cAMP-dependent kinase (PKA), a highly homologous kinase. The X-ray crystal structure of ROCK bound to ATP-competitive inhibitors revealed two kinase domains linked by an N-terminal dimerisation domain comprised of five α-helices from each monomer. In this arrangement, the active sites of the two kinases share a common face, possibly facilitating interactions with dimeric substrates or inhibitory domains. Each kinase domain appears to have a catalytically competent conformation in the absence of phosphorylation. Comparison of the four ROCK-ligand structures - both among themselves and against their corresponding counterparts in PKA - suggested that a very specific active site contact is important for selective ROCK inhibition.[12]

In the present study we did not define protein–ligand complex structures of ROCK with our novel Y-27632 derivatives **10** and **11**. However, our functional evaluation in two hPS cell lines suggest that 3-fluoro substitution in the pyridine ring in **11** interferes with the active site contact of ROCK, resulting in a diminished biological activity *i.e.* >50% compared to Y-27632 (**1**). This effect is even more pronounced in derivative **10**, when fluorine is substituted in the 2-position complete loss of biological activity was provoked.

Conclusions

In summary, the present work discloses a practical and scalable synthesis of the ROCK inhibitor Y-27632 (**1**) with complete characterisation by ^{1}H-, ^{13}C-NMR- and IR-spectroscopy, MS- and HRMS-spectrometry and m.p. as well as specific optical rotation. The title compound was obtained in 45% yield over seven steps. The key hydrogenation step afforded the *trans*-(*R*)-4-(1-aminoethyl) cyclohexane carboxylic acid **5a** as a single isomer. The optimised synthesis also served to access two new fluoro derivatives. Biological evaluation of new derivatives **10** and **11** in human pluripotent stem cells revealed that fluorine substitution in the pyridine ring partially or completely diminished the supporting effect of the original molecule Y-27632 on hPSC vitality and expansion after dissociation.

Experimental

General remarks

All solvents were dried by conventional methods. Starting materials and reagents were purchased from commercial suppliers and used without further purification. Preparative column chromatography was performed using silica gel 60, particle size 0.040–0.063 mm (230–240 mesh, flash). Analytical TLC was

carried out employing silica gel 60 F254 plates from Merck, Darmstadt. Visualisation of the developed chromatograms was performed with detection by UV (254 nm), by colouration with a phosphomolybdic acid solution in EtOH or ninhydrin solution in EtOH. NMR spectra were recorded on a Bruker ARX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. All spectra were measured using standard Bruker pulse sequences. Mass spectra were obtained from a Micromass LCT *via* loop-mode injection from a Waters (Alliance 2695) HPLC system. HRMS data were recorded on a Micromass Q-TOF in combination with a Waters Aquity Ultraperformance LC system. Ionisation was achieved by ESI or APCI. Melting points were measured on a SRS OptiMelt apparatus and are uncorrected. Optical rotation was measured with a Perkin Elmer 341 polarimeter. IR spectra were recorded with a Bruker Vektor 22 FT-IR spectrophotometer (GoldenGate ATR unit).

(*R*)-*N*-(1-Phenylethyl)acetamide

Acetic anhydride (33.7 g, 330 mmol, 2 equiv.) was added dropwise to a solution of (*R*)-1-phenylethylamine (**2**) (20 g, 165 mmol, 1 equiv) in chloroform (150 mL) at 0 °C. After completion of the reaction (checked by TLC, ethyl acetate/methanol, 9 : 1), ice water (150 mL) was added and the mixture was extracted with chloroform (3 × 150 mL). The extracts were washed with 1 N aqueous solution of NaOH (100 mL), dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried overnight under vacuum to afford the title compound (25.6 g, 156.8 mmol; 95%) as colourless crystals.

mp 100–101 °C (ref. 13*a* mp 99–100 °C, ref. 13*b* mp 97–100 °C); $[\alpha]_D^{20}$ +140.4 (*c* 1, $CHCl_3$) [ref. 3*c* $[\alpha]_D$ +143.5 (*c* 1, EtOH), ref. 13*a* $[\alpha]_D$ +138.8 (*c* 2.35, EtOH), ref. 13*b* $[\alpha]_D$ +141.0 (*c* 1, $CHCl_3$)]. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.50 (d, 3H, $^3J_{HH}$ = 6.9 Hz, CH_3), 2.01 (s, 3H, CH_3CONH), 5.21 (quint, 1H, $^3J_{HH}$ = 6.9 Hz, CH), 5.83 (bs, 1H, NH), 7.31–7.39 (m, 5H, 5 × Ar-H);

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 21.7 (CH_3), 23.5 (CH_3), 48.8 (CH), 126.2 (Ar-CH), 127.4 (Ar-CH), 128.7 (Ar-CH), 143.1 (Ar-C), 169.0 (C═O); MS (+ESI): *m*/*z* (%) = 186.0822 (90) $[M+Na]^+$, 164.0918 (100) $[M+H]^+$; HR-MS: 164.1068 (164.1075 calc. for $C_{10}H_{14}N_1O_1$).

The spectroscopic data of (*R*)-*N*-(1-phenylethyl)acetamide were in full agreement with those reported in the literature.[3c,13]

(*R*)-*N*-(1-(4-Acetylphenyl)ethyl)acetamide

Aluminium chloride (44 g, 331.3 mmol, 2 equiv.) was added portionwise to a solution of (*R*)-*N*-(1-phenylethyl)acetamide (27 g, 165.6 mmol, 1 equiv.) in 1,2-dichloroethane (150 mL) at r.t. Then, acetyl chloride (15.6 g, 198.7 mmol, 1.2 equiv.) was added dropwise. The mixture was stirred at r.t. for 1 h and subsequently at 60 °C for 3 h. After completion of the reaction (checked by TLC, ethyl acetate), the mixture was poured onto ice water and extracted with dichloromethane (3 × 150 mL). The extracts were washed with water (100 mL) dried over $MgSO_4$, filtered, concentrated and the residue obtained was crystallised (isopropyl ether/ethanol 9 : 1) to afford the title compound (30.6 g, 149.1 mmol; 90%) as orange crystals. mp 125–126 °C; $[\alpha]_D^{20}$ +173.8 (*c* 0.5, $CHCl_3$) [ref. 3*c* $[\alpha]_D$ +162.0 (*c* 1, MeOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.49 (d, 3H, $^3J_{HH}$ = 7.0 Hz, CH_3), 1.99 (s, 3H, CH_3CONH), 2.56 (s, 3H, CH_3CO), 5.14 (quint, 1H, $^3J_{HH}$ = 7.0 Hz, CH), 5.90 (bs, 1H, NH), 7.41 (d, 2H, $^3J_{HH}$ = 8.3 Hz, 2 × Ar-H), 7.92 (d, 2H, $^3J_{HH}$ = 8.3 Hz, 2 × Ar-H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 21.8 (CH_3), 23.3 (CH_3), 26.6 (CH_3), 48.6 (CH), 126.3 (Ar-CH), 128.5 (Ar-CH), 136.1 (Ar-C), 148.7 (Ar-C), 169.2 (C═O), 197.7 (C═O); MS (+ESI): *m*/*z* (%) = 228.0920 (75) $[M+Na]^+$, 206.0948 (100) $[M+H]^+$; HR-MS: 206.1176 (206.1186 calc. for $C_{12}H_{16}N_1O_2$).

The spectroscopic data of (*R*)-*N*-(1-acetylphenylethyl)-acetamide were in full agreement with those reported in the literature.[3c,13d]

(*R*)-4-(1-Acetamidoethyl)benzoic acid (3)

Sodium hypochlorite (12% solution, 130 mL) was slowly added to a mixture of (*R*)-*N*-(1-(4-acetylphenyl)ethyl)acetamide (10 g, 48.7 mmol, 1 equiv.), and sodium hydroxide (2.2 g, 52.9 mmol, 1.1 equiv.) in methanol (100 mL) at r.t. The mixture was heated to 60 °C for 2 h. After completion of the reaction (checked by TLC, ethyl acetate/methanol 9 : 1), the solvent was removed and the residue obtained was poured onto ice water (200 mL). Then, conc. hydrochloride acid was added which resulted in a yellow crystalline precipitate. This was filtered off and the acidic aqueous filtrate was extracted with ethyl acetate (3 × 100 mL). The organic layers were combined and evaporated, dried overnight under vacuum which gave another crop of yellow crystals which was combined with the yellow crystalline material obtained before to yield the title compound **3** (9.18 g, 44.3 mmol; 91%). mp decomposition at about 175 °C; $[\alpha]_D^{20}$ +116.7 (*c* 1, MeOH) [ref. 3*c* $[\alpha]_D$ +136.8 (*c* 1, MeOH). ^{1}H-NMR (MeOH-d_4, 400 MHz): δ 1.49 (d, 3H, $^3J_{HH}$ = 7.1 Hz, CH_3), 2.01 (s, 3H, CH_3CONH), 5.06 (q, 1H, $^3J_{HH}$ = 7.1 Hz, CH), 7.49 (d, 2H, $^3J_{HH}$ = 8.2 Hz, 2 × Ar-H), 8.01 (d, 2H, $^3J_{HH}$ = 8.2 Hz, 2 × Ar-H). The signals for N*H* and COO*H* could not be detected in the spectrum; ^{13}C-NMR (MeOH-d_4, 100 MHz): δ 23.1 (CH_3), 23.4 (CH_3), 50.9 (CH), 127.9 (Ar-CH), 131.4 (Ar-C), 131.5 (Ar-CH), 151.5 (Ar-C), 170.5 (C═O), 173.3 (C═O); MS (+ESI): *m*/*z* (%) = 230.0850 (30) $[M+Na]^+$, 208.0799 (100) $[M+H]^+$; HR-MS: 208.0949 (208.0974 calc. for $C_{11}H_{14}N_1O_3$).

The spectroscopic data of (*R*)-4-(1-acetamidoethyl)benzoic acid (**3**) were in full agreement with those reported in the literature.[3c]

Catalytic hydrogenation – general procedure

A solution of benzoic acid **3** and 5% ruthenium on carbon in 26% aqueous ammonia was stirred in an autoclave. Then, the catalyst was filtered off through a short pad of Celite™. The filter pad was washed with water and the filtrate was concentrated and dried overnight under vacuum to afford 1,4-*trans* **4a** and *ca.* 10–20% of starting material **3** (method A), a mixture of 1,4-*trans* **4a** and 1,4-trans **5a** in varying ratios (method B) or only 1,4-*trans* **5a** (method C).

Method A: *trans*-(*R*)-4-(1-Acetamidoethyl)cyclohexane carboxylic acid (4a)

Benzoic acid **3** (1 g, 4.83 mmol) and 5% ruthenium on carbon (0.7 g) in 26% aqueous ammonia (5 mL) were reacted for 6 h at 90 °C and for 6 h at 120 °C according to the general procedure. The initial hydrogen pressure was set at 50 bar. *Trans*-**4a** (0.80 g, 78%) was obtained which was contaminated with the starting material **3** (*ca* 10%, judged by ^{1}H NMR spectroscopy).

Method B: mixture of *trans*-4a and *trans*-5a

Benzoic acid **3** (5 g, 24.1 mmol) and 5% ruthenium on carbon (5 g) in 26% aqueous ammonia (25 mL) were reacted for 3 h at 90 °C and then for 1–3 d at 150 °C according to the general procedure. The initial hydrogen pressure was set at 70 bar. A mixture of *trans*-**4a** and *trans*-**5a** (3–3.9 g, calc. *ca* 69–80%) was obtained.

Method C: *trans*-(*R*)-4-(1-Aminoethyl)cyclohexanecarboxylic acid (*trans*-5a)

Benzoic acid **3** (5 g, 24.1 mmol) and 5% ruthenium on carbon (5 g) in 26% aqueous ammonia (25 mL) were reacted at 90 °C for 3 h and then at 150 °C for 3 d to the according to the general procedure. The initial hydrogen pressure was set at 70 bar. 1,4-*Trans* **5a** (2.89 g, 16.9 mmol; 70%) was obtained.

Transformation of the mixture of 1,4-*trans* 4a and 1,4-*trans* 5a

A solution of a mixture of **4a** and **5a** (4 g) obtained from insufficient hydrogenation in 26% aqueous ammonia (40 mL) was stirred under a hydrogen atmosphere (70 bar) in an autoclave at 160 °C for 3 d. Then, the solvent was evaporated and the product was dried overnight under vacuum to exclusively afford the carboxylic acid **5a** (3.7 g).

4a: In all instances **4a** contained traces of benzoic acid **3** as judged from NMR-analysis.

^{1}H-NMR (MeOH-d_4/DMSO-d_6, 400 MHz): δ 1.05 (d, 3H, $^3J_{HH}$ = 6.8 Hz, CH_3), 1.17–1.68 (m, 7H), 1.92 (s, 3H, CH_3CO), 2.05 (m, 2H), 2.52 (m, 1H, H-1), 3.81 (quint, 1H, $^3J_{HH}$ = 6.8 Hz, $CHCH_3$). The signals for NH and COOH were not detected in the spectrum.

^{13}C-NMR (MeOH-d_4/DMSO-d_6, 100 MHz): δ 16.9 (CH_3), 21.4 (CH_3CO), 25.5 (CH_2), 25.6 (CH_2), 26.1 (CH_2), 26.3 (CH_2), 39.5 (CH-4), 41.7 (CH-1), 47.8 ($CHCH_3$), 170.8 (CH_3CO), 177.2 (C═O); MS (–ESI): m/z (%) = 212.1570 (100) $[M-H]^-$; MS (+ESI): m/z (%) = 277.1996 (80) $[M+ CH_3CN + Na]^+$, 213.1982 (100) $[M]^+$; mixture of acetonitrile/H_2O was used as a solvent; HR-MS: 277.1529 (277.1528 calc. for $C_{11}H_{19}N_1O_3 + CH_3CN + Na^+$); mixture of acetonitrile/H_2O was used as a solvent.

5a: mp 255 °C [ref. 5 mp >290 °C]; $[\alpha]_D^{20}$ +0.4 (*c* 0.5, H_2O). ^{1}H-NMR (D_2O, 400 MHz): δ 1.16 (m, H-3a and H-5a), 1.18 (d, 3H, $^3J_{HH}$ = 6.7 Hz, CH_3), 1.37 (m, 1H, H-4), 1.45 (dq, 2H, $^3J_{HH}$ = 3.4 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-2a or H-6a), 1.46 (dq, 2H, $^3J_{HH}$ = 3.4 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-6a or H-2a), 2.01 (m, 2H, H-3e and H-5e), 2.05 (m, 2H, H-2e and H-6e), 2.18 (tt, 1H, $^3J_{HH}$ = 3.5 Hz, $^3J_{HH}$ = 12.3 Hz, H-1), 2.88 (quint, 1H, $^3J_{HH}$ = 6.4 Hz, $CHCH_3$). The signals for NH_2 and COOH were not detected in the spectrum; ^{13}C-NMR (D_2O, 100 MHz): δ 18.9 (CH_3), 28.1 (CH_2), 28.3 (CH_2), 30.2 (2 × CH_2), 43.7 (CH-4), 47.6 (CH-1), 51.4 ($CHCH_3$), 186.8 (C═O); MS (+ESI): m/z (%) = 172.1392 (100) $[M+H]^+$.

***trans*-(*R*)-4-(1-Aminoethyl)cyclohexanecarboxylic acid hydrochloride (7a)**

Concentrated HCl (0.5 mL) was slowly added to a solution of 1,4-*trans* **5a** (0.3 g, 1.44 mmol) in water (2 mL). The mixture was stirred overnight, the solvent was evaporated and the resulting solid dried overnight under vacuum and then recrystallised from a mixture of ethanol/acetonitrile 1 : 1 to afford an analytically pure sample of the title compound **7a** (0.28 g, 1.35 mmol; 76.9%). mp 254 °C [ref. 5 mp >290 °C]; $[\alpha]_D^{20}$ +7.5 (*c* 0.5, MeOH). ^{1}H-NMR (D_2O, 400 MHz): δ 1.16 (dq, 2H, $^3J_{HH}$ = 3.1 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-3a and H-5a), 1.26 (d, 3H, $^3J_{HH}$ = 6.8 Hz, CH_3), 1.42 (dq, 1H, $^3J_{HH}$ = 3.1 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-2a or H-6a), 1.43 (dq, 1H, $^3J_{HH}$ = 3.1 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-6a or H-2a), 1.59 (dtt, 1H, $^3J_{HH}$ = 3.1 Hz, $^3J_{HH}$ = 6.5 Hz, $^3J_{HH}$ = 12.6 Hz, H-4), 1.83 (m, 2H, H-3e and H-5e), 2.05 (m, 2H, H-2e and H-6e), 2.35 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 12.3 Hz, H-1), 3.22 (quint, 1H, $^3J_{HH}$ = 6.5 Hz, $CHCH_3$). The signals for NH_2 and COOH were not detected in the spectrum; ^{13}C-NMR (D_2O, 100 MHz): δ 14.9 (CH_3), 26.1 (CH_2), 27.2 (CH_2), 27.8 (CH_2), 27.9 (CH_2), 39.9 (CH-4), 42.5 (CH-1), 51.9 ($CHCH_3$), 181.0 (C═O); MS (+ESI): m/z (%) = 172.1407 (100) $[M+H]^+$; HR-MS: 172.1334 (172.1338 calc. for $C_9H_{18}N_1O_2$).

***trans*-(*R*)-4-(1-Aminoethyl)cyclohexanecarboxylic acid methyl ester (6a)**

Methanolic HCl (1.25 N solution, 9.3 mL, 11.7 mmol, 2 equiv.) was added to cyclohexanecarboxylic acid **5a** (1 g, 5.84 mmol, 1 equiv.) in dry methanol (20 mL) under an argon atmosphere. The mixture was heated under refluxing conditions for 2 h. Then, the solvent was removed under vacuum and chloroform (30 mL) and water (30 mL) were added. After addition of solid potassium carbonate (1 g) the mixture was extracted with chloroform (3 × 30 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound **6a** (0.86 g, 4.64 mmol; 80%) as an oil which was used without further purification in the isomerisation experiment.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.00 (m, 2H, H-3a and H-5a), 1.01 (d, 3H, $^3J_{HH}$ = 6.5 Hz, CH_3), 1.12 (m, 1H, H-4), 1.39 (dq, 1H, $^3J_{HH}$ = 3.1 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-2a or H-6a), 1.40 (dq, 1H, $^3J_{HH}$ = 3.1 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-6a or H-2a), 1.50 (bs, 2H, NH_2), 1.81 (m, 2H, H-3e and H-5e), 1.99 (m, 2H, H-2e and H-6e), 2.19 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 12.3 Hz, H-1), 2.65 (quint, 1H, $^3J_{HH}$ = 6.5 Hz, $CHCH_3$), 3.62 (s, 3H, $COOCH_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 20.9 (CH_3), 27.8 (CH_2), 27.9 (CH_2), 28.8 (CH_2), 27.9 (CH_2), 43.2 (CH-4), 44.4 (CH-1), 51.2 ($COOCH_3$), 51.4 ($CHCH_3$), 176.4 (C═O); MS (+ESI): m/z (%) = 186.1375 (100) $[M+H]^+$; HR-MS: 186.1488 (186.1494 calc. for $C_{10}H_{20}N_1O_2$).

Isomerisation attempts with the acids 5a and 6a

Potassium *tert*-butoxide (0.1 g, 0.88 mmol, 3 equiv.) was added to a solution of cyclohexanecarboxylic acid **5a** (0.05 g, 0.29 mmol, 1 equiv.) in ethanol (2 mL) and the mixture was heated under refluxing conditions for 2 d. Then, the mixture was concentrated and the residue was dried overnight under vacuum. The starting material **5a** was completely recovered as judged by ^{1}H- and ^{13}C-NMR spectroscopy.

Likewise, potassium *tert*-butoxide (0.09 g, 0.81 mmol, 3 equiv.) was added to a solution of methyl ester **6a** (0.05 g, 0.27 mmol, 1 equiv.) in ethanol (2 mL) and the mixture was heated under refluxing conditions for 2 d. Then, the mixture was concentrated and the residue dried overnight under vacuum to quantitatively afford the acid **5a** as was judged by ^{1}H- and ^{13}C-NMR spectroscopy.

trans-(*R*)-4-(1-(*tert*-Butoxycarbonylamino)ethyl) cyclohexanecarboxylic acid (8)

Cyclohexanecarboxylic acid **5a** (5 g, 29.2 mmol, 1 equiv.) was dissolved in a mixture of water (150 mL) and THF (150 mL) at 0 °C. Then, sodium hydroxide (2.35 g, 58.5 mmol, 2 equiv.) was added and after stirring for 30 min, di-*tert*-butyl dicarbonate (7.65 g, 35.1 mmol, 1.2 equiv.) was added. The mixture was warmed to r.t and stirred for 2 d (progress was checked by TLC, ethyl acetate/petroleum ether 1 : 1). THF was removed under reduced pressure, the aqueous layer was acidified with HCl (1 N) to pH 3-4 and extracted with ethyl acetate (3 × 150 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product **8** was purified by flash column chromatography (SiO_2, ethyl acetate/petroleum ether 1 : 2; R_f = 0.42) to afford the Boc-protected acid **8** (7 g, 25.8 mmol; 89%) as colourless crystals. mp 106–108 °C; $[\alpha]_D^{20}$ +0.8 (*c* 0.6, MeOH). ^{1}H-NMR (MeOH-d_4, 400 MHz): δ 1.08 (m, 2H, H-3a and H-5a), 1.10 (d, 3H, $^3J_{HH}$ = 6.8 Hz, CH_3), 1.32 (m, 3H, H-2a, H-4 and H-6a), 1.47 (s, 9H, $C(CH_3)_3$), 1.88 (m, 2H, H-3e and H-5e), 2.05 (m, 2H, H-2e and H-6e), 2.22 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 12 Hz, H-1), 3.40 (quint, 1H, $^3J_{HH}$ = 6.8 Hz, $CHCH_3$). The signals of NH and COOH were not detected in the spectrum. ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 18.3 (CH_3), 28.4 ($(CH_3)_3$), 27.7 (CH_2), 28.0 (CH_2), 28.5 (2 × CH_2), 42.7 (CH-4), 43.0 (CH-1), 50.4 ($CHCH_3$), 79.1 (C-$(CH_3)_3$), 155.5 (NHC═O), 181.6 (C═O); MS (–ESI): *m*/*z* (%) = 270.1447 (100) $[M-H]^-$; HR-MS: 270.1705 (270.1705 calc. for $C_{14}H_{24}N_1O_4$).

trans-tert-Butyl (*R*)-1-[(1-(1*R*,4*R*)-4-(pyridine-4-ylcarbamoyl)cyclohexyl]ethylcarbamate (9)

Procedure according to Mukaiyama. 1-Methyl-2-chloropyridinium iodide (5.65 g, 22.1 mmol, 1.2 equiv.) was added to a stirred mixture of 8 (5 g, 18.5 mmol, 1 equiv.), 4-aminopyridine (1.8 g, 19.4 mmol, 1.05 equiv.) and triethylamine (6.3 mL, 44.2 mmol, 2.4 equiv.) in dichloromethane (250 mL) under an argon atmosphere at r.t. The reaction was stirred for 2 d (checked by TLC, dichloromethane/methanol 95 : 5 and ethyl acetate/petroleum ether 1 : 1). After completion of the reaction, water (250 mL) was added and the mixture was extracted with dichloromethane (3 × 200 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2; dichloromethane/methanol 95 : 5; R_f = 0.15) to afford the title compound 9 (6.2 g, 17.8 mmol; 97%) as yellow crystals.

TBTU-method. DIPEA (0.1 mL, 0.55 mmol, 1.5 equiv.) and TBTU (0.14 g, 0.44 mmol, 1.2 equiv.) were successively added to a stirred mixture of 8 (0.1 g, 0.37 mmol, 1 equiv.), 4-aminopyridine (0.04 g, 0.41 mmol, 1.1 equiv.) in dichloromethane (5 mL) under an argon atmosphere at 0 °C. The reaction mixture was warmed to r.t. and checked by TLC (dichloromethane/methanol 95 : 5 and ethyl acetate/petroleum ether 1 : 1). After stirring overnight, water (5 mL) was added and the mixture extracted with dichloromethane (3 × 4 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2; dichloromethane/methanol 95 : 5; R_f = 0.15) to afford the title compound 9 (0.11 g, 0.32 mmol; 85%) as yellow crystals. mp 182 °C; $[\alpha]_D^{20}$ +4.5 (*c* 0.7, MeOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.07 (m, 2H, H-3a and H-5a), 1.08 (d, 3H, $^3J_{HH}$ = 6.7 Hz, CH_3), 1.42 (s, 9H, $C(CH_3)_3$), 1.43 (m, 3H, H-2a, H-4, H-6a), 1.85 (m, 2H, H-3e and H-5e), 2.02 (m, 2H, H-2e and H-6e), 2.33 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 12 Hz, H-1), 3.48 (quint, 1H, $^3J_{HH}$ = 6.7 Hz, $CHCH_3$), 4.48 (bs, 1H, NH), 7.59 (d, 2H, $^3J_{HH}$ = 7.2 Hz, 2 × Py-H), 8.44 (d, 2H, $^3J_{HH}$ = 7.2 Hz, 2 × Py-H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 18.6 (CH_3), 28.4 ($(CH_3)_3$), 28.4 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 31.8 (CH_2), 38.6 (CH), 42.7 (CH), 46.2 (CH), 79.1 (C-$(CH_3)_3$), 113.6 (Py-CH), 145.6 (Py-C), 150.4 (Py-CH), 155.6 (NHC═O), 175.3 (C═O); MS (+ESI): *m*/*z* (%) = 348.2146 (100) $[M+H]^+$; HR-MS: 348.2288 (348.2287 calc. for $C_{19}H_{30}N_3O_3$).

Rho-Kinase inhibitor Y-27632 (1)

A hydrogen chloride solution (1.0 N in diethyl ether, 202 mL, 202 mmol, 10 equiv.) was added dropwise at 0 °C to a stirred mixture of amide **9** (7 g, 20.2 mmol, 1 equiv.) in dichloromethane (200 mL) under an argon atmosphere. The reaction was warmed up to r.t. and stirred for 2 d (progress was checked by TLC, dichloromethane/methanol 1 : 1). After completion of the reaction, the solvents were evaporated, dried overnight under vacuum and recrystallised from a mixture of diethyl ether/methanol 9 : 1 to afford the title compound **1** (6.1 g, 19.1 mmol; 95%) as yellowish crystals. mp 258 °C [ref. 3*b* mp 286–287 °C for bis-hydrochloride monohydrate and 276 °C for bis-hydrochloride $\frac{1}{2}$ hydrate]; $[\alpha]_D^{20}$ –11.5 (*c* 0.5, MeOH) [ref. 3*b* $[\alpha]_D$ +4.6 (*c* 1, MeOH); ref. 4*b* $[\alpha]_D$ +4.3 (*c* 1, MeOH)]. IR (film): $\tilde{\nu}$ (cm^{-1}) = 2858, 1713, 1639, 1609, 1573, 1502, 1480, 1386, 1312, 1292, 1246, 1176, 1139, 1039, 929, 82; ^{1}H-NMR (MeOH-d_4, 400 MHz): δ 1.27 (dq, 1H, $^3J_{HH}$ = 3.4 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-3a or H-5a), 1.28 (dq, 1H, $^3J_{HH}$ = 3.4 Hz, $^2J_{HH}$ = $^3J_{HH}$ = 13 Hz, H-5a or H-3a), 1.33 (d, 3H, $^3J_{HH}$ = 6.8 Hz, CH_3), 1.62 (m, 3H, H-2a, H-4, CH-6a), 1.95 (m, 2H, H-3e and H-5e), 2.13 (m, 2H, H-2e and H-6e), 2.57 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 11.9 Hz, H-1), 3.20 (quint, 1H, $^3J_{HH}$ = 6.8 Hz, $CHCH_3$), 8.25 (d, 2H, $^3J_{HH}$ = 7.2 Hz, 2 × Py-H), 8.64 (d, 2H, $^3J_{HH}$ = 7.2 Hz, 2 × Py-H). The signals for NH and NH_2 were not detected in the spectrum; ^{13}C-NMR (MeOH-d_4, 100 MHz): δ 16.8 (CH_3), 28.5 (CH_2), 29.8 (CH_2), 30.3 (CH_2), 30.4 (CH_2), 42.7 (CH-4), 47.5 (CH-1), 54.0 ($CHCH_3$), 116.7 (Py-CH), 144.0 (Py-CH), 156.4 (Py-C), 178.9 (C═O); MS (+ESI): *m*/*z* (%) = 248.1512 (100) $[M+H]^+$; MS (–ESI): *m*/*z* (%) = 282.1715 (100) $[M+Cl]^-$; HR-MS: 248.1763 (248.1763 calc. for $C_{14}H_{22}N_3O_1$).

The spectroscopic data of the Rho-Kinase inhibitor Y-27632 (**1**) were in full agreement with those recorded for an authentic sample.[11]

(*R*)-*trans*-4-(1-aminoethyl)-*N*-(2-fluoro-4-pyridyl)-cyclohexanecarboxamide dihydrochloride (10)

1-Methyl-2-chloropyridinium iodide (0.09 g, 0.34 mmol, 1.2 equiv.) was added at r.t to a stirred mixture of **8** (0.075 g, 0.28 mmol, 1 equiv.), 2-fluoro-4-aminopyridine (0.03 g, 0.29 mmol, 1.05 equiv.) and triethylamine (0.09 mL, 0.67 mmol, 2.4 equiv.) in dichloromethane (5 mL) under an argon atmosphere. The reaction was stirred for 2 d (checked by TLC, ethyl acetate/petroleum ether 1 : 1). After completion of the reaction, water (5 mL) was added and the mixture was extracted with dichloromethane (3 × 5 mL). The combined organic layers were dried over

$MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2; ethyl acetate/petroleum ether 1 : 1; R_f = 0.33) to afford the Boc-protected amide (0.04 g, 0.11 mmol; 39%) as a viscous oil.

A hydrogen chloride solution (1.0 N in diethyl ether, 1 mL, 1.0 mmol, 10 equiv.) was added dropwise at 0 °C to a stirred mixture of Boc-protected amide (0.035 g, 0.1 mmol, 1 equiv.) in dichloromethane (2 mL) under an argon atmosphere. The reaction was warmed up to r.t. and stirred for 2 d (progress was checked by TLC, dichloromethane/methanol 1 : 1). After completion of the reaction, the solvents were evaporated, the crude product was dried overnight under vacuum and recrystallised from a mixture of diethyl ether/methanol 99 : 1 to afford amide **10** (0.03 g, 0.09 mmol; 94%) as colourless crystals. mp decomposition at 88–118 °C; $[\alpha]_D^{20}$ –4.8 (*c* 0.25, MeOH). IR (film): $\tilde{\upsilon}$ (cm^{-1}) = 2933, 1680, 1596, 1503, 1413, 1328, 1254, 1171, 1121, 1096, 1002, 970, 929, 903, 856, 740; ^{1}H-NMR (MeOH-d_4, 400 MHz): δ 1.25 (m, 2H, H-3a and H-5a), 1.31 (d, 3H, $^3J_{HH}$ = 6.8 Hz, CH_3), 1.60 (m, 3H, H-2a, H-4, CH-6a), 1.92 (m, 2H, H-3e and H-5e), 2.06 (m, 2H, H-2e and H-6e), 2.44 (tt, 1H, $^3J_{HH}$ = 3.4 Hz, $^3J_{HH}$ = 12.0 Hz, H-1), 3.18 (quint, 1H, $^3J_{HH}$ = 6.5 Hz, *CH*CH_3), 7.42 (dt, 1H, $^3J_{HH}$ = 5.8 Hz, $^4J_{HH}$ = 1.4 Hz, Py-H), 7.54 (d, 1H, $^4J_{HH}$ = 1.4 Hz, Py-H), 8.08 (d, 1H, $^3J_{HH}$ = 5.8 Hz, Py-H). The signals for NH and NH_2 were not detected in the spectrum; ^{13}C-NMR (MeOH-d_4, 100 MHz): δ 15.9 (CH_3), 27.7 (CH_2), 29.1 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 41.9 (CH-4), 46.5 (CH-1), 53.2 (*C*HCH_3), 99.4 (d, Py-CH, $^2J_{CF}$ = 41.5 Hz), 113.0 (d, Py-CH, $^4J_{CF}$ = 3.6 Hz), 148.1 (d, Py-CH, $^3J_{CF}$ = 15.4 Hz), 152.5 (d, Py-C, $^3J_{CF}$ = 12.2 Hz), 165.8 (d, Py-C, $^1J_{CF}$ = 234.9 Hz), 177.7 (C═O); MS (–ESI): *m*/*z* (%) = 264.1606 (100) $[M-H]^-$; HR-MS: 264.1517 (264.1512 calc. for $C_{14}H_{19}N_3O_1F_1$).

(*R*)-*trans*-4-(1-aminoethyl)-*N*-(3-fluoro-4-pyridyl)-cyclohexanecarboxamide dihydrochloride (11)

1-Methyl-2-chloropyridinium iodide (0.14 g, 0.55 mmol, 1.2 equiv.) was added at r.t to a stirred mixture of **8** (0.125 g, 0.46 mmol, 1 equiv.), 3-fluoro-4-aminopyridine (0.05 g, 0.48 mmol, 1.05 equiv.) and triethylamine (0.15 mL, 1.10 mmol, 2.4 equiv.) in dichloromethane (6 mL) under an argon atmosphere. The reaction was stirred for 2 d (checked by TLC, ethyl acetate/petroleum ether 1 : 1). After completion of the reaction, water (6 mL) was added and the mixture was extracted with dichloromethane (3 × 6 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2; ethyl acetate/petroleum ether 1 : 1; R_f = 0.51) to afford the Boc-protected amide (0.085 g, 0.23 mmol 50%) as yellowish crystals.

A hydrogen chloride solution (1.0 N in diethyl ether, 2.2 mL, 2.2 mmol, 10 equiv.) was added dropwise at 0 °C to a stirred mixture of Boc-protected amide (0.08 g, 0.22 mmol, 1 equiv.) in dichloromethane (4 mL) under an argon atmosphere. The reaction was warmed up to r.t. and stirred for 2 d (progress was checked by TLC, dichloromethane/methanol 1 : 1). After completion of the reaction, the solvents were evaporated, the crude product was dried overnight under vacuum and recrystallised from a mixture of diethyl ether/methanol 9 : 1 to afford the amide **11** (0.07 g, 0.21 mmol; 95%) as yellow crystals. mp decomposition at 180–219 °C; $[\alpha]_D^{20}$ –6.4 (*c* 0.9, MeOH). IR (film): $\tilde{\upsilon}$ (cm^{-1}) = 2933, 1715, 1640, 1605, 1657, 1488, 1387, 1313, 1245, 1121, 931, 904, 825, 756. ^{1}H-NMR (MeOH-d_4, 400 MHz): δ 1.32 (m, 5H, H-3a, H-5a and CH_3), 1.65 (m, 3H, H-2a, H-4, CH-6a), 1.94 (m, 2H, H-3e and H-5e), 2.10 (m, 2H, H-2e and H-6e), 2.72 (m, 1H, H-1), 3.19 (m, 1H, *CH*CH_3), 8.59 (m, 1H, Py-H), 8.98 (m, 2H, 2 × Py-H). The signals for NH and NH_2 were not detected in the spectrum. ^{13}C-NMR (MeOH-d_4, 100 MHz): δ 16.9 (CH_3), 28.5 (CH_2), 29.8 (CH_2), 30.6 (CH_2), 30.7 (CH_2), 42.6 (CH-4), 47.0 (CH-1), 54.1 (*C*HCH_3), 118.2 (Py-CH), 132.8 (d, Py-CH, $^2J_{CF}$ = 30.5 Hz), 142.4 (Py-CH), 145.1 (Py-C), 150.4 (Py-C, $^1J_{CF}$ = 249.0 Hz), 178.9 (C═O); MS (–ESI): *m*/*z* (%) = 264.1559 (100) $[M-H]^-$; HR-MS: 264.1516 (264.1512 calc. for $C_{14}H_{19}N_3O_1F_1$); MS (+ESI): *m*/*z* (%) = 266.1399 (100) $[M+H]^+$; HR-MS: 266.1661 (266.1669 calc. for $C_{14}H_{21}N_3O_1F_1$).

In vitro assessment of 1, 10 and 11

The cell lines hES3 (ES Cell International, Singapore[2c]) and hCBiPS2, (LEBAO, MHH Hannover[2a]) were applied for in vitro functional testing, respectively. For conventional 2D cultures, cells were grown on γ-irradiated human foreskin fibroblasts or mouse embryonic fibroblasts in KO medium (KnockOut DMEM, 20% KnockOut Serum Replacement, 50 ng/ml bFGF, 2mM L-glutamine, and 1% MEM Non Essential Amino Acids; Invitrogen) and passaged every 4–7 days.

Feeder-based hESC or hiPSC cultures were dissociated into single cells by collagenase B (Roche) treatment and inoculated in mTeSR medium (STEMCELL Technologies Inc.) at 100.000 cells per 3 ml in low attachment 6 well dishes to generate suspension cultures (culture set up was described in detail by Zweigerdt *et al.*[2a]). Cells were cultured for 4 days (one passage) without medium change either in mTeSR alone (controls) or supplemented with 10 μM of **1**, **10** or **11**, respectively. On day four, aggregate formation was monitored by light microscopy. For subsequent passaging, aggregates were dissociated into single cells by collagenase B treatment and re-inoculated at the initial density of 100.000 cells per 3 ml medium per well. Cell yields were analysed by Trypan blue staining for cell vitality and cell counting was performed using a Haemocytometer; 3 parallel wells were counted for each condition; the cell yield was analysed for 3 independent passages applying supplementation with the respective compound.

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