Synthesis and evaluation of novel pyridine based PLG tripeptidomimetics†

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Analogues of the pyridine based PLG (Pro-Leu-Gly-NH_2) peptidomimetic **1** were synthesized and evaluated as dopamine modulating agents. Modifications in the position corresponding to the leucine side chain in PLG afforded derivatives **2**, **3** and **4**, substituted with H, Me and Bn instead of the isobutyl group, respectively. Changes in the proline residue produced derivative **5**, substituted with a symmetrical piperidine ring instead of the pyrrolidine ring and **6**, in which the pyrrolidine ring is connected to the pyridine ring *via* a hydroxymethyl group instead of a keto function. The peptidomimetics were tested for their ability to enhance the maximal effect of *N*-propylapomorphine (NPA) at dopamine D2 receptors in the functional cell-based R-SAT assay. Compounds **2**, **3**, and **4**, produced a statistically significant increase in the maximal NPA response at 10 nM (117 ± 6%, 118 ± 6%, and 116 ± 3%, respectively), which is similar to the effect of PLG in this assay, whereas **5** was able to potentiate the response to a similar extent at 1 nM concentration (115 ± 5%). All derivatives produced a bell-shaped dose–response curve and none of the compounds were active at the D2 receptor alone, which indicates that the mechanism behind the activity of both the pyridine based mimetics **1–6** and PLG is the same. Interestingly, L-Pro-D-Leu-Gly-NH_2 was found to be more potent than PLG and produced a 119 ± 1% increase in the NPA response at 1 nM.

Introduction

PLG (Pro-Leu-Gly-NH_2) is an endogenous peptide found in the central nervous system.[1] It is known to exert its pharmacological effect(s) through the modulation of dopamine D2 receptors.[2,3] Previous studies have shown that PLG enhances the effect of dopamine agonists at D2 receptors in a biphasic dose-dependent manner to give a bell-shaped dose–response curve.[4] A number of active PLG mimetics, ranging from peptide analogues[5–12] to constrained peptidomimetics,[5,13–23] have been presented in the literature. Recently, we reported the development of a 2,3,4-substituted pyridine scaffold for tripeptidomimetics and its use in the synthesis of **1**, a potent PLG mimetic as found in the functional R-SAT assay (Fig. 1).[24]

In this study, peptidomimetics **2–6** were designed in order to further investigate the structural requirements for the dopamine modulating effects of compounds based on the pyridine scaffold (Fig. 2). Studies using peptide analogues and peptidomimetics of PLG have shown that variations in the side chain of the leucine residue can have a profound effect on the mimicking ability.[8,18,21] Therefore, the synthesis and evaluation of compounds **2**, **3** and **4**, in which the isobutyl side chain of leucine was replaced with a hydrogen, a methyl or a benzyl group, respectively, was undertaken. Modifications in the proline residue of PLG have also been shown to have a significant influence on the activity of PLG analogues.[7] Two analogues of **1** with modified proline residues were investigated, the achiral piperidine derivative **5** and the alcohol **6**.

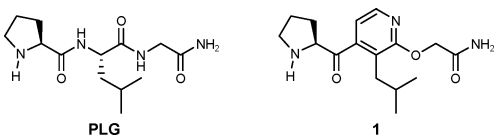

Fig. 1 PLG and the pyridine based peptidomimetic **1**.

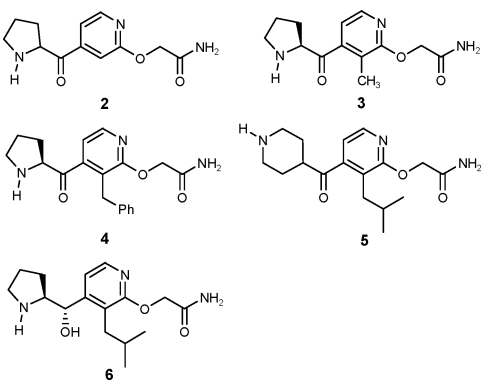

Fig. 2 Synthesized and evaluated analogues of **1**.

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† Electronic supplementary information (ESI) available: NMR-spectra of biologically evaluated compounds, and synthetic procedures and characterization data for compounds **3**, **5**, **6**, **9b**, **10b**, **11b**, **12b**, **15**, **16**, **18**, **19**, **20**, **21** and **22**. See DOI: 10.1039/b718058f

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Results and discussion

Synthesis

Tripeptidomimetics **2**, **3**, **4** and **5** were synthesized according to a previously published strategy for **1**.[24] The synthesis is based on a functionalized pyridine scaffold with the side-chain of the second amino acid of the tripeptide attached to the 3-position of the pyridine ring, and with fluorine and iodine as handles in the neighboring 2- and 4-positions, respectively (Fig. 3). The *N*- and *C*-terminal residues are attached to the functionalized scaffold *via* a Grignard reaction utilizing the iodine handle, and a nucleophilic substitution reaction of fluorine, respectively.

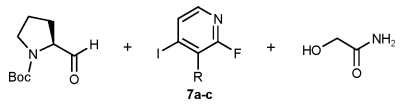
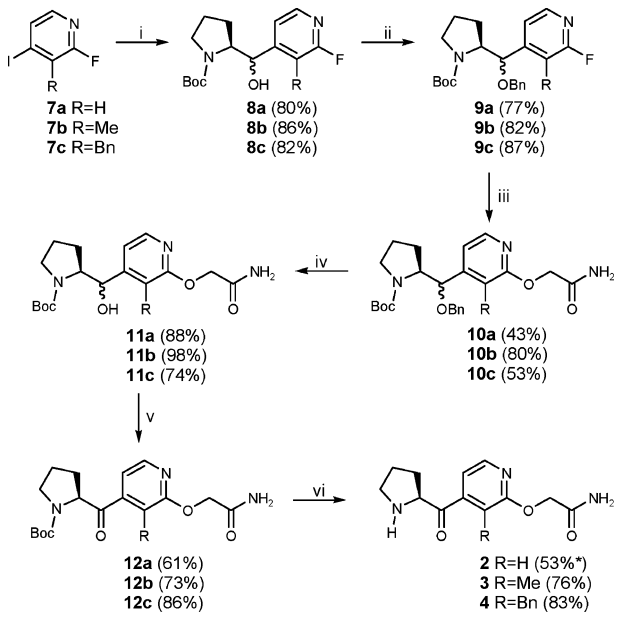


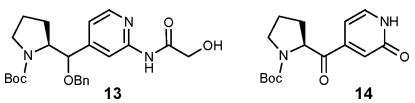
Fig. 3 Building blocks for assembly of the pyridine based peptidomimetics **2**, **3** and **4**.

The synthesis of the PLG mimetics with modifications in the Leu-residue, compounds **2**, **3** and **4**, is described in Scheme 1. The functionalized scaffolds **7a–c** were synthesized from 2-fluoropyridine in 71–89% yield as previously described.[25] Attachment of Boc-protected proline aldehyde *via* a Grignard reaction using *i*PrMgCl to accomplish an iodine–magnesium exchange in the 4-position of the pyridine ring proceeded in 80–86% yield. To allow careful control of each reaction step using NMR-spectroscopy the diastereomeric alcohols of **8c** (dr 1 : 9, calculated on isolated yields) were separated, and the reaction sequence continued with the major isomer. As no problems were identified in the synthesis of ketone **4** it was considered most efficient to avoid separation of the diastereomeric alcohols in the synthesis of the H- and Me-derivatives **2** and **3**. Thus, in the synthesis of **8a** and **8b**, the isomeric mixtures were not separated and the subsequent four reaction steps were performed on the diastereomeric mixtures.



Scheme 1 Synthesis of mimetics **2**, **3** and **4** modified at the residue corresponding to leucine. *Reagents and conditions*: (i) *i*PrMgCl, Boc-Pro-CHO, THF, rt; (ii) BnBr, NaH, cat. Bu_4NI, THF, rt; (iii) glycolamide, KH, DMSO, 55 °C; (iv) H_2, cat. Pd/C, THF or EtOAc, rt; (v) Dess–Martin periodinane, CH_2Cl_2, rt; (vi) TFA, CH_2Cl_2, rt.

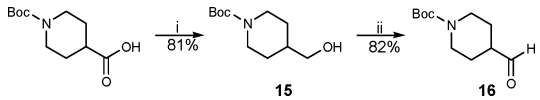
Protection of the secondary alcohols as benzyl ethers was accomplished by reaction with sodium hydride and benzyl bromide to provide **9a–c** in 77–87% yield. Introduction of the *C*-terminal glycolamide moiety was performed using KH as the base in DMSO at 55 °C, in a nucleophilic aromatic substitution reaction. The reaction proceeded in good to moderate yield for the methyl- and benzyl-derivatives to afford **10b** and **10c** (80 and 53%, respectively). A different reactivity was observed for **9a** which lacks a substituent *ortho* to the site of substitution. Here the reaction with glycolamide was found to be complete after only two hours, as compared to days for the other derivatives, and two products, the wanted **10a** and the byproduct **13** were isolated in almost equal amounts, 43% and 48%, respectively. Attempts to improve the selectivity by performing the reaction at a lower temperature (room temperature) were not successful. The formation of a byproduct resulting from the nucleophilic attack of the amide instead of the alcohol functionality has previously been reported for the corresponding 3-methallyl derivative when the reaction was run at a higher temperature (80 °C).[24] However, the selectivity barrier for **9a** seems to be considerably lower than for the 3-substituted derivatives.



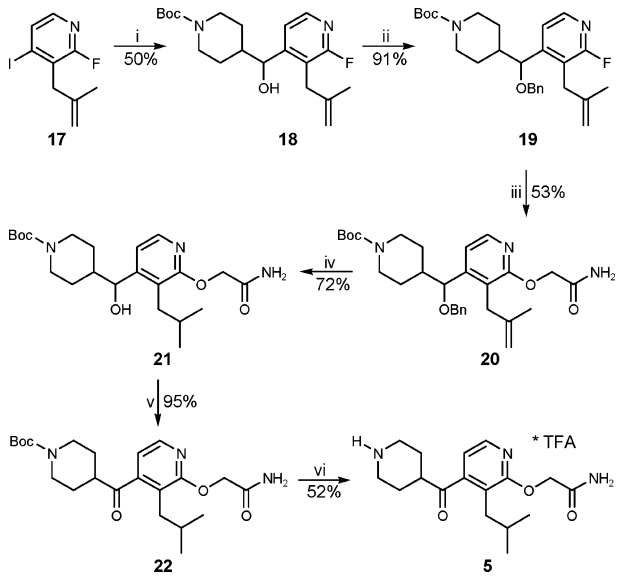
The secondary alcohols **10a–c** were deprotected by catalytic hydrogenation with Pd/C to afford **11a–c** in 74–98% yield. Oxidation of the secondary alcohols using Dess–Martin periodinane in CH_2Cl_2 provided **12a–c** in 61–86% yield. Also in this step, the unsubstituted derivative **12a** was found to differ from the others. When allowing the reaction to run overnight, **12a** was isolated in a poor 21% yield from a mixture with the 2-pyridone **14**. The formation of this byproduct could however be avoided simply by shortening the reaction time to one hour, whereby the yield of **12a** was improved to 61%. In the final step, Boc-deprotection of the pyrrolidine nitrogen was accomplished by treatment with 25% trifluoroacetic acid in CH_2Cl_2 for 5–7 minutes, which allowed **2**, **3** and **4** to be isolated in 53–83% yield after purification by reversed-phase preparative HPLC. During the characterization of the TFA salt of **2** by NMR spectroscopy in MeOH-d_4, it was found to exist in equilibrium between the keto and enol tautomers. The formation of the enol could actually be followed during the ^1H NMR spectral analysis, where the peaks corresponding to the enol form increased in intensity over time at the expense of the keto isomer, until an approximately 1 : 1 ratio was obtained. By analysis of the free amine in $CDCl_3$, no keto isomer could be detected, instead an equilibrium between the *E*- and *Z*-enol isomers was observed. This finding was also supported by the lack of optical activity of **2**. The reason for this unusual behavior of the unsubstituted derivative has not been studied in more detail, but a possible explanation could be that the lack of a substituent at the 3-position of the pyridine ring permits coplanarity between the keto–enol functionality and the aromatic ring which favors formation of an enol. The 3-substituted derivatives **3** and **4** did not show the same tautomerization in a polar solvent. The optical

activity of the methyl derivative **3** was followed over time in MeOH, and it was found to be stable over 3 days at $[\alpha]_D = -37$.

Aldehyde **16**, required for the synthesis of peptidomimetic **5**, was obtained from *N*-Boc-isonipecotic acid by reduction to the alcohol **15** (81% yield) followed by oxidation with Dess–Martin periodinane (82% yield) (Scheme 2). The aldehyde was used directly in the subsequent Grignard reaction. The synthesis of **5** was accomplished *via* a route similar to that described for **2–4** above (Scheme 3).



Scheme 2 Synthesis of *N*-Boc-isonipecotic aldehyde **16**. *Reagents and conditions*: (i) isobutylchloroformate, *N*-methylmorpholine, $NaBH_4$, THF, −20 °C; (ii) Dess–Martin periodinane, CH_2Cl_2, rt.



Scheme 3 Synthesis of mimetic **5** with a modified residue corresponding to proline. *Reagents and conditions*: (i) *i*PrMgCl, **16**, THF, rt; (ii) BnBr, NaH, cat. Bu_4NI, THF, rt; (iii) glycolamide, KH, DMSO, 55 °C; (iv) H_2, cat. Pd/C, rt; (v) Dess–Martin periodinane, CH_2Cl_2, rt; (vi) TFA, CH_2Cl_2, rt.

Peptidomimetic **6** was synthesized in 56% yield from the corresponding Boc-protected alcohol[24] by treatment with TFA–CH_2Cl_2 as described above.

Pharmacology

The PLG mimetics synthesized were evaluated in the R-SAT assay, a pharmacologically predictive cell-based functional assay based on the ligand-dependent transformation of NIH/3T3 cells as previously described.[24,26] The compounds were tested for their ability to enhance the maximum response of the dopamine agonist *N*-propylapomorphine (NPA) at the human D2 receptor (Fig. 4). All derivatives showed a bell-shaped dose–response curve. A statistically significant enhancement of the NPA response was produced by compounds **2** (117 ± 6%), **3** (118 ± 5%) and **4** (116 ± 3%), all at 10 nM. These activities were comparable to the enhancement produced by PLG in the R-SAT assay (115 ± 6% at 10 nM).[24] Compound **5** was found to produce its highest effect at 1 nM (115 ± 5%), whereas the response by compound **6** was not statistically significant due to its large variability (114 ± 7%; $p > 0.05$). In addition to the synthesized compounds, the peptide L-Pro-D-Leu-Gly-NH_2 (**23**) was evaluated as a dopamine potentiator and found to produce a significant enhancement in the NPA response at both 0.1 nM (116 ± 3%) and 1 nM (119 ± 1%) (Fig. 4).

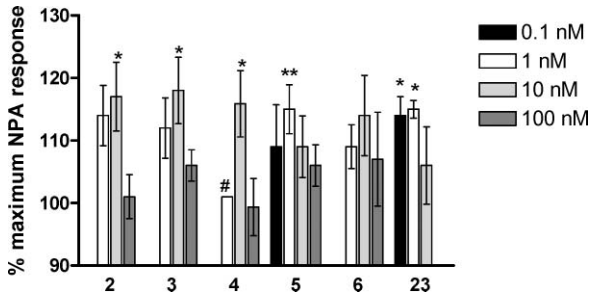


Fig. 4 Potentiation of the NPA response at the D2 receptor by mimetics **2**, **3**, **4**, **5**, **6** and **23**. Data represent the percentage of the maximum NPA response for NPA alone, achieved in the presence of the respective mimetic. Shown is the average and standard error of between three and ten separate experiments, each carried out 12 times. Paired *t*-tests indicate significant differences from the values for NPA alone (* = $p < 0.05$; ** = $p < 0.01$). # $n = 1$.

Compounds **1–6** were also tested for agonist activity at the D2 receptor. The dopamine agonist NPA was used as a positive control. None of the peptidomimetics were active at the D2 receptor at concentrations between 0.01 and 10 μM. The results for mimetics **1**, **5** and **6** are shown in Fig. 5.

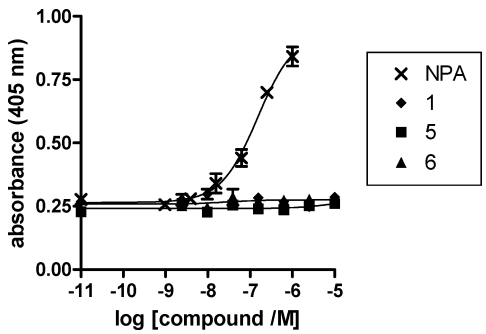


Fig. 5 Agonist activity of **1**, **5** and **6** and the reference compound NPA at the D2 receptor.

All of the evaluated pyridine based PLG mimetics produced bell-shaped dose–response curves, and none were active as agonists at the dopamine D2 receptor. These findings suggest that the mechanism behind the activity of peptidomimetics **1–6** and PLG is the same. However, the functional assay that has been used in this study gives no information regarding the binding site(s), so it is not known whether peptidomimetics **1–6** bind to the same site at the D2 receptor as PLG, or not.

Earlier studies with peptide analogues of PLG modified at the leucine residue have shown that a hydrophobic residue in this position is a prerequisite for activity.[8] Substitution of the isobutyl group of PLG with benzyl or *n*-butyl afforded derivatives with activities comparable to that of PLG in enhancing the binding

of the dopamine agonist ADTN to dopamine receptors, whereas substitution with smaller alkyl moieties, propyl or ethyl, yielded inactive analogues. This trend cannot be found in our series of pyridine based PLG mimetics. The differences among **1**, **2**, **3**, and **4** are too small to enable a ranking based on their activities, and they are therefore considered to be of equal potency. It can be speculated that the pyridine scaffold itself might be able to interact with a hydrophobic pocket in the receptor, thereby reducing the importance of such an interaction by the side chain moiety. However, attention should also be given to the racemization of **2** *via* keto–enol tautomerism and the possibility that the two enantiomers differ in activity.[7]

Earlier studies have shown that the proline residue of PLG can be quite sensitive to structural modifications. For example, a peptide analogue with a thiazolidine-4-carbonyl group instead of a proline residue was found to be inactive, whereas the pyroglutamyl derivative of PLG showed an activity comparable to that of the parent peptide.[7] In our series, the activity of the piperidine derivative **5** is considered most interesting since it shows that significant changes in the proline moiety can be accepted in the pyridine based PLG mimetics. It is also the only pyridine based PLG mimetic that is active at 1 nM, where the increase in the NPA response was similar to that produced by PLG at 10 nM. In analogue **5**, the symmetrical piperidine ring makes the compound achiral, which simplifies synthetic and stability considerations. Also, the nitrogen atom in the 4-substituted piperidine ring of **5** is positioned quite differently from the nitrogen atom in the 2-substituted pyrrolidine ring of **1**. The acceptance of this replacement by the PLG binding site adds interesting information to the discussion about the bioactive conformation of PLG, which has been postulated in several studies to be a type II β-turn.[13–17] In the previous study of mimetic **1**, we concluded that **1** could not adopt a type II β-turn conformation, and speculated about the possibility of an extended conformation as a second bioactive conformation of PLG.[24] This hypothesis has been suggested by others,[5] and is supported by the activity of **5**, which is predicted to adopt extended conformations to an even greater extent than **1**, due to the 180° constraint imposed by the *para*-relationship of the peptide backbone through the piperidine ring. Alcohol **6** produced an enhancement of the NPA response that was not statistically significant and no conclusions about the outcome of the bioisosteric replacement of the keto-functionality of **1** with an alcohol in **6** can therefore be drawn.

In the design of the pyridine scaffold, it was noticed that the direction of the side chain moiety that is attached to the 3-position of the pyridine ring, was close to the direction of a D-amino acid in the corresponding position of the parent peptide. The established ability of mimetic **1** to mimic the action of PLG therefore suggested that the PLG diastereomer L-Pro-D-Leu-Gly-NH_2 (**23**) could be active as a dopamine modulator. Although several tripeptide derivatives have been evaluated as PLG mimetics, most with an L-configuration in the second amino acid moiety,[8] to our knowledge no ^{2}D-isomer of an ^{2}L-dopamine modulating active tripeptide has been reported. The hypothesis of the pyridine scaffold as an L-D-L tripeptidomimetic, was therefore challenged by the inclusion of L-Pro-D-Leu-Gly-NH_2 (**23**) in our test series. Most interestingly, **23** was found to be more potent than PLG, and produced a similar response at 1 nM as PLG did at a 10 nM concentration (Fig. 4).

Conclusion

Five novel PLG mimetics based on a 2,3,4-trisubstituted pyridine scaffold have been synthesized and four of them, plus the peptide L-Pro-D-Leu-Gly-NH_2, were found to have dopamine modulatory activity in the R-SAT assay comparable to (**2**, **3**, and **4**), or higher (**5** and **23**), than PLG. None of the mimetics showed any agonistic activity at the D2 receptor, and all produced bell-shaped dose–response curves similar to that of PLG. These findings suggest a similar mechanism behind the activity of both PLG and the pyridine based peptidomimetics **1–6** and provide additional information towards the elucidation of the bioactive conformation of PLG.

Experimental section

Chemistry. General data

All chemical reagents were commercially available. THF was distilled from potassium/sodium. TLC analysis was performed on silica gel F_{254} (Merck) and detection was carried out by examination under UV light and staining with phosphomolybdic acid. After work-up, all organic phases were dried with $MgSO_4$. Flash column chromatography was performed on silica gel with solvent of HPLC grade or analytical grade. Analytical reversed-phase HPLC was performed on a Beckman System Gold HPLC equipped with a Kromasil C-8 column (250 × 4.6 mm) using acetonitrile (0.1% TFA) in H_2O (0.1% TFA), 0–100% linear gradient over 60 minutes. A flow-rate of 1.5 mL min^{-1} was used and detection was at 254 nm. Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 20 mm) using the same eluent, a flow rate of 11 mL min^{-1} and detection at 254 nm. Chiral HPLC chromatography was performed on a Pirkle Covalent (*S*,*S*)-Whelk-O 1 10/100 Krom Fec column using CH_2Cl_2–iPrOH–heptane (48 : 4 : 48) as the eluent, a flow rate of 1.0 mL min^{-1} and detection at 254 nm. Melting points were determined on a Büchi melting point apparatus and are uncorrected. Specific rotations were measured on a Perkin-Elmer model 343 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer at 400 and 100 MHz, respectively, and calibrated using the residual peak of solvent as an internal reference ($CDCl_3$ [$CHCl_3$ δ_H 7.26, $CDCl_3$ δ_C 77.0] or CD_3OD [CD_2HOD δ_H 3.34, CD_3OD δ_C 49.0]) or DMSO-d_6 [δ_H 2.50, δ_C 39.52]. Infrared spectra were recorded using an ATI Mattson Infinity Series FTIR spectrometer. High-resolution mass spectral analysis was performed at Instrumentstationen, Lund University, Sweden. Compounds **7a**,[27] **7b**,[27] **7c**,[25] **8b**[25] and **17**[25] were synthesized as reported earlier. The tripeptide **23** was commercially available (Bachem). Data on compounds **3**, **5**, **6**, **9b**, **10b**, **11b**, **12b**, **15**, **16**, **18**, **19**, **20**, **21**, and **22** and selected NMR-spectra are presented in the ESI†.

General procedure for the Grignard coupling reactions to produce 8a, 8c and 18. The pyridine derivative (1.0 eq.) was dissolved in freshly distilled THF and isopropyl magnesium chloride (1.0 eq.) was added as a 2.0 M solution in THF. The reaction was allowed to stir for 1–2 h at room temperature under a nitrogen atmosphere, before the aldehyde (1.0 eq.) was added as a solution in THF. The reaction was stirred overnight before it was quenched by the addition of H_2O and extracted with diethyl ether. The combined

organic phases were washed with brine, dried and concentrated to yield a crude oil, which was purified as specified below.

(1*R*,1*S*)-[(2*S*)-1-*tert*-Butoxycarbonylpyrrolidin-2-yl]-1-(2-fluoro-pyridin-4-yl)-methanol (8a). The general procedure applied to pyridine derivative **7a** (0.50 g, 2.24 mmol) and Boc-Pro-CHO in THF (5 mL, total volume) yielded a crude oil, which was purified by flash chromatography using CH_2Cl_2–MeOH–heptane (5 : 1: 10) as the eluent to afford **8a** (0.53 g, 80%) as a colorless oil: ^{1}H NMR ($CDCl_3$) (mixture of isomers) δ 8.17–8.12 (m, 1H), 7.17–7.11 (m, 1H), 6.97–6.91 (m, 1H), 6.12 (br s, 0.8H), 5.44 (br s, 0.2H), 5.24–4.51 (m, 1H), 4.30–3.79 (m, 1H), 3.69–3.18 (m, 2H), 2.06–1.57 (m, 4H), 1.60–1.35 (m, 9H); ^{13}C NMR ($CDCl_3$) (mixture of isomers) δ 163.79 (d, J = 239 Hz), 157.75 (br), 157.40 (d, J = 7 Hz), 147.20 (d, J = 14 Hz), 119.98, 107.89 (d, J = 39 Hz), 80.98, 76.37 (br), 63.21, 62.88, 47.59, 28.85, 28.48, 28.27, 27.84, 23.70; IR (neat) 3391 (br), 2974, 2938, 2887, 1690, 1668, 1613, 1409 cm^{-1}; HRMS (FAB) calcd for $C_{15}H_{22}FN_2O_3$ $[M + H]^+$ 297.1614, found 297.1612.

(1*R*,1*S*)-(3-Benzyl-2-fluoro-pyridin-4-yl)-1-[(2*S*)-1-*tert*-butoxy-carbonylpyrrolidin-2-yl]-methanol (8c major and 8c minor). The general procedure applied to pyridine derivative **7c** (0.94 g, 3.0 mmol) and Boc-Pro-CHO in THF (15 mL, total volume) yielded a crude oil, which was purified by flash chromatography using heptane–EtOAc (6 : 1) as the eluent to afford the diastereomers **8c major** (0.57 g, 74%) and **8c minor** (62 mg, 8%) as pale yellow oils: **8c major**: ^{1}H NMR ($CDCl_3$) δ 8.11 (d, 1H, J = 5.0 Hz), 7.33–7.11 (m, 6H), 6.23 (br s, 1H), 4.84 (dd, 1H, J = 9.0, 2.6 Hz), 4.23–4.03 (m, 3H), 3.46–3.33 (m, 1H), 3.27–3.18 (m, 1H), 1.90–1.44 (m, 4H), 1.52 (s, 9H); ^{13}C NMR ($CDCl_3$); δ 162.60 (d, J = 239 Hz), 159.67, 154.90 (br), 145.68 (d, J = 16 Hz), 138.64, 128.64, 128.24, 126.49, 120.51, 120.32 (d, J = 30 Hz), 81.29, 76.41, 73.94, 63.42, 47.62, 30.64, 28.69, 23.76; IR (neat) 3317, 2976, 2932, 2882, 1658, 1606, 1404, 1163; HRMS (FAB) calcd for $C_{22}H_{28}FN_2O_3$ $[M + H]^+$ 387.2084, found 387.2084; $[\alpha]_D$ +22 (*c* 1.0, $CHCl_3$). **8c minor**: IR (neat) 3356, 2976, 2881, 2360, 1679, 1607, 1401, 1256, 1164, 1114; $[\alpha]_D$ −48 (*c* 1.0, $CHCl_3$); HRMS (FAB) calcd for $C_{22}H_{28}FN_2O_3$ $[M + H]^+$ 387.2084, found 387.2083.

General procedure for the benzyl protections to produce 9a, 9b, 9c and 19. The alcohol (1.0 eq.) was dissolved in THF and stirred while NaH (1.5 eq.) was added carefully, followed by benzyl bromide (1.5 eq.) and tetrabutylammonium iodide (0.05 eq.). The reaction was stirred at room temperature overnight before it was quenched by the addition of NH_4Cl (aq., sat.) and extracted with diethyl ether. The combined organic phases were washed with brine, dried and concentrated to yield a crude oil, which was purified as specified below.

4-[(1*R*,1*S*)-Benzyloxy-1-((2*S*)-1-*tert*-butoxycarbonyl-pyrrolidin-2-yl)-methyl]-2-fluoro-pyridine (9a). The general procedure applied to alcohol **8a** (0.37 g, 1.24 mmol) in THF (5 mL) yielded a crude oil, which was purified by flash chromatography using EtOAc–heptane (1 : 10) as the eluent to afford **9a** (0.37 g, 77%, diastereomeric mixture) as a colorless oil: ^{1}H NMR ($CDCl_3$) (mixture of isomers) δ 8.22–8.13 (m, 1H), 7.38–7.20 (m, 5H), 7.16–7.04 (m, 1H), 7.02–6.88 (m, 1H), 5.20–5.07 (m, 0.5H), 4.87–4.75 (m, 0.5H), 4.68–4.75 (m, 2.7H), 3.99–3.82 (m, 0.3H), 3.62–3.17 (m, 1.3H), 2.96–2.77 (m, 0.7H), 2.12–1.85 (m, 2H), 1.85–1.31 (m, 10H), 1.07–0.87 (m, 1H); ^{13}C NMR ($CDCl_3$) (mixture of isomers) δ 164.20 (d, J = 239 Hz), 163.99 (d, J = 239 Hz), 155.99, 154.94, 154.65, 154.37, 153.99, 147.81, 147.63, 147.47, 147.16, 137.78, 137.46, 128.43, 127.96, 127.75, 127.46, 120.25 (d, J = 3 Hz), 119.38 (d, J = 3 Hz), 108.08 (d, J = 37 Hz), 107.15 (d, J = 38 Hz), 79.99, 79.76, 79.45, 79.35, 78.54, 72.55, 72.33, 72.05, 71.59, 62.38, 60.49, 60.26, 47.42, 47.19, 46.96, 46.81, 28.46, 26.29, 25.58, 25.32, 24.55, 24.42, 23.59, 22.91; IR 3065, 3031, 2974, 2880, 1692, 1611, 1567, 1453, 1369, 1273, 1172, 1085 cm^{-1}; HRMS (FAB) calcd for $C_{22}H_{28}FN_2O_3$ $[M + H]^+$ 387.2084, found 387.2087.

3-Benzyl-4-[benzyloxy-1-((2*S*)-1-*tert*-butoxycarbonyl-pyrrolidin-2-yl)-methyl]-2-fluoro-pyridine (9c). The general procedure applied to alcohol **8c major** (0.75 g, 1.9 mmol) in THF (5 mL) yielded a crude oil, which was purified by flash chromatography using EtOAc–heptane (1 : 4) as the eluent to afford **9c** (0.62 g, 87%) as a brownish viscous oil: ^{1}H NMR ($CDCl_3$) (rotamers) δ 8.18–8.04 (m, 1H), 7.44 (s, 1H), 7.39–7.02 (m, 10H), 5.11 (br s, 0.5H), 4.78 (br s, 0.5H), 4.48–3.89 (m, 5H), 3.59–2.92 (m, 2H), 2.11–1.75 (m, 2H), 1.76–1.04 (m, 11H); ^{13}C NMR ($CDCl_3$) δ 162.51 (d, J = 240 Hz), 155.32, 152.94, 145.48, 145.29, 145.05, 144.89, 138.80, 138.35, 137.73, 137.41, 128.62, 128.30, 127.52, 126.45, 122.08, 120.61, 79.73, 78.15, 71.41, 60.72, 46.89, 30.14, 28.47, 28.15, 25.94, 24.01, 23.33; IR (neat) 2957, 2923, 2855, 1690, 1389, 1258, 1091, 1014 cm^{-1}; $[\alpha]_D$ −20 (*c* 1.0, $CHCl_3$); HRMS (FAB) calcd for $C_{29}H_{33}FN_2O_3$ $[M + H]^+$ 477.2553, found 477.2554.

General procedure for the nucleophilic substitution reactions to produce 10a, 10b, 10c and 20. Glycolamide (5.0 eq.) was dissolved in DMSO (pre-dried with 4 Å molecular sieves, $\frac{1}{2}$ of the total volume) and KH (5.0 eq.) was added carefully. After a few minutes, the evolution of gas stopped and the pyridine derivative (1.0 eq.) was added as a solution in DMSO ($\frac{1}{2}$ of the total volume). The reaction was stirred at the temperature, and for the time, specified below before it was quenched by the addition of NH_4Cl (aq., sat.) and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried and concentrated to yield a crude oil, which was purified as specified below.

2-{4-[(1*R*,1*S*)-Benzyloxy-1-((2*S*)-1-*tert*-butoxycarbonyl-pyrrolidin-2-yl)-methyl]-pyridin-2-yloxy}-acetamide (10a). The general procedure was applied to pyridine derivative **9a** (0.33 g, 0.85 mmol) in DMSO (2.5 mL total volume). The reaction was stirred at 55 °C for 2 hours. The work-up procedure yielded a crude oil, which was purified by flash chromatography using EtOAc–heptane (1: 1, then 2 : 1) as the eluent to afford the diastereomers **10a** (0.16 g, 43%) and the byproduct **13** (0.18 g, 48%) as colorless oils: **10a**: ^{1}H NMR ($CDCl_3$) (mixture of isomers and rotamers) δ 8.15–8.07 (m, 1H), 7.39–7.23 (m, 5H), 7.06–6.97 (m, 0.2H), 6.94–6.77 (m, 1.8H), 6.42 (br s, 1H), 5.96 (br s, 1H), 5.13–4.95 (m, 0.5H), 4.83 (s, 2H), 4.79–4.70 (m, 0.5H), 4.68–3.82 (m, 3H), 3.59–2.78 (m, 2H), 2.11–1.66 (m, 3H), 1.58–1.32 (m, 9H), 1.11–0.90 (m, 1H); ^{13}C NMR ($CDCl_3$) (mixture of isomers and rotamers) δ 171.70, 162.32, 162.14, 154.73, 154.46, 154.31, 153.90, 153.14, 152.95, 152.14, 151.83, 146.90, 146.75, 146.56, 146.30, 138.04, 137.91, 137.55, 128.31, 128.22, 127.66, 127.45, 127.30, 127.13, 117.07, 116.13, 109.12, 108.20, 79.96, 79.70, 79.42, 79.17, 78.65, 72.11, 71.91, 71.62, 71.21, 64.39, 62.29, 62.17, 60.37, 60.15, 47.27, 46.97, 46.81, 46.59, 28.35, 26.14, 25.43, 25.27, 24.40, 24.29, 23.52, 23.43, 22.79; IR 3332, 3061, 2976, 2881, 1682, 1613, 1560, 1417, 1306,

1164, 1119, 1061 cm^{-1}; HRMS (FAB) calcd for $C_{24}H_{32}N_3O_5$ [M + H]$^+$ 442.2342, found 442.2345. **13**: ^{1}H NMR ($CDCl_3$) (mixture of isomers and rotamers) δ 9.51–9.39 (m, 1H), 8.41–8.27 (m, 1H), 8.26–8.16 (m, 1H), 7.39–7.25 (m, 5H), 7.19–6.99 (m, 1H), 6.41–5.75 (br s, 1H), 5.10–3.84 (m, 6H), 3.57–2.83 (m, 2H), 2.12–1.81 (m, 2H), 1.79–1.29 (m, 10H), 1.14–0.96 (m, 1H); ^{13}C NMR ($CDCl_3$) (mixture of isomers and rotamers) δ 170.89, 170.65, 154.91, 154.54, 154.13, 153.06, 152.36, 151.84, 150.95, 150.88, 150.79, 147.38, 147.04, 146.88, 146.72, 137.92, 137.64, 128.43, 128.33, 128.22, 127.77, 127.59, 127.47, 119.00, 117.90, 113.14, 112.15, 112.06, 80.34, 79.99, 79.82, 79.63, 79.31, 79.15, 72.26, 72.05, 71.81, 71.39, 62.46, 62.28, 62.11, 60.61, 47.37, 46.95, 46.69, 28.45, 28.37, 26.35, 25.62, 25.35, 24.75, 24.29, 23.65, 22.93; IR 3370, 3063, 2976, 2881, 1682, 1566, 1519, 1403, 1169, 1080 cm^{-1}; HRMS (FAB) calcd for $C_{24}H_{32}N_3O_5$ [M + H]$^+$ 442.2342, found 442.2342.

2-{3-Benzyl-4-[benzyloxy-1-((2*S*)-1-*tert*-butoxycarbonyl-pyrrolidin-2-yl)-methyl]-pyridin-2-yloxy}-acetamide (10c). The general procedure was applied to pyridine derivative **9c** (0.42 g, 0.88 mmol) in DMSO (12 mL total volume). The reaction was stirred at room temperature for one week and at 55 °C for one day. An additional 5 eq. of pre-reacted glycolamide and KH were added and the reaction was stirred for 5 days at 55 °C. The work-up procedure described above yielded a crude oil, which was purified by flash chromatography using EtOAc–heptane (1 : 1) as the eluent to afford recovered starting material (0.10 g, 24%), and **10c** (0.25 g, 53%) as a white foam: $[\alpha]_D$ −10 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) (rotamers); δ 8.14–8.04 (m, 1H), 7.36–7.11 (m, 9H), 7.06–6.98 (m, 2H), 5.55–5.25 (m, 2H), 5.21–4.80 (m, 2H), 4.63–4.15 (m, 4H), 4.15–3.93 (m, 2H), 3.50–2.95 (m, 2H), 2.14–1.78 (m, 2H), 1.73–1.04 (m, 11H); ^{13}C NMR ($CDCl_3$) (rotamers) δ 171.49, 160.13, 155.22,154.63, 149.86, 144.66, 144.37, 140.16, 139.65, 137.83, 137.57, 128.71, 128.32, 127.55, 126.30, 121.61, 121.09, 117.00, 79.52, 77.64, 71.51, 71.33, 64.16, 60.86, 46.87, 46.53, 30.89, 28.41, 28.15, 27.39, 25.92, 23.95, 23.12; IR (neat) 3020, 1215 cm^{-1}; HRMS (FAB) calcd for $C_{31}H_{38}N_3O_5$ [M + H]$^+$ 532.2811, found 532.2814.

General procedure for the catalytic hydrogenation reactions to produce 11a, 11b, 11c and 21. The benzyl ether (1.0 eq.) was dissolved in the solvent specified below and Pd/C (10 wt%) was added. The reaction was stirred under H_2 at room temperature for the time specified below, before the catalyst was filtered off using a plug of Celite® and the filtrate was concentrated to afford an oil, which was either pure or was purified as specified below.

2-{4-[1-((2*S*)-1-*tert*-Butoxycarbonyl-pyrrolidin-2-yl)-(1*R*,1*S*)-hydroxy-methyl]-pyridin-2-yloxy}-acetamide (11a). The general procedure was applied to benzyl ether **10a** (0.15 g, 0.34 mmol) and Pd/C (75 mg) in THF (4 mL). The reaction was stirred over two days, with additional Pd/C (75 mg) being added twice. A precipitate was formed that could not be removed by filtration through Celite® but had to be separated from the product either by flash chromatography, or by dissolving the crude brown mixture in EtOAc and performing the filtration procedure again. Work-up yielded a brown liquid, which was purified by flash chromatography EtOAc–heptane (2 : 1 then 4 : 1 then 6 : 1) to afford **11a** (105 mg, 88%) as a colorless oil: ^{1}H NMR ($CDCl_3$) (mixture of isomers) δ 8.08–8.01 (m, 1H), 6.94–6.78 (m, 2H), 6.51 (br s, 1H), 6.35 (br s, 1H), 6.06 (br s, 0.5H), 5.26 (br s, 0.2H), 5.11 (br s, 0.1H), 4.99–4.89 (m, 0.3H), 4.83–4.71 (m, 2H), 4.65–4.45 (m, 0.6H), 4.23–3.85 (m, 1H), 3.59–2.91 (m, 2H), 2.08–1.13 (m, 13H); ^{13}C NMR ($CDCl_3$) (mixture of isomers) δ 171.80, 171.76, 162.21, 157.97, 154.83, 146.74, 146.42, 116.96, 116.49, 109.13, 108.60, 80.98, 77.20, 74.35, 64.44, 63.35, 62.89, 47.84, 47.62, 28.34, 28.12, 26.62, 23.78; IR 3350 (br), 2976, 2882, 1674, 1613, 1408, 1167, 1061 cm^{-1}; HRMS (FAB) calcd for $C_{17}H_{26}N_3O_5$ [M + H]$^+$ 352.1872, found 352.1870.

2-{3-Benzyl-4-[1-((2*S*)-1-*tert*-butoxycarbonyl-pyrrolidin-2-yl)-hydroxy-methyl]-pyridin-2-yloxy}-acetamide (11c). The general procedure was applied to **10c** (0.11 g, 0.21 mmol) and Pd/C (51 mg) in THF (5 mL). The reaction was stirred for two hours. Work-up, including purification by flash chromatography (CH_2Cl_2–MeOH–heptane 4 : 1 : 10), afforded **11c** (56 mg, 74%) as a white foam: $[\alpha]_D$ +6.0 (*c* 0.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.08 (d, 1H, J = 5.0 Hz), 7.30–7.07 (m, 6H), 6.18 (br s, 1H), 5.46–5.31 (m, 2H), 4.93 (d, 1H, J = 16 Hz), 4.87 (d, 1H, J = 8.7 Hz), 4.65 (d, 1H, J = 16 Hz), 4.28–4.04 (m, 3H), 3.49–3.25 (m, 2H), 1.74–1.17 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 171.45, 160.23, 158.35, 152.08, 144.97, 139.76, 128.70, 127.95, 126.38, 120.39, 117.14, 81.16, 74.71, 64.21, 63.40, 47.62, 31.40, 30.27, 28.52, 28.36, 23.85; HRMS (FAB) calcd for $C_{24}H_{32}N_3O_5$ [M + H]$^+$ 442.2342, found 442.2350.

General procedure for the oxidation reactions to produce 12a, 12b, 12c and 22. The alcohol (1.0 eq.) was dissolved in CH_2Cl_2 and Dess–Martin periodinane (15 wt% in CH_2Cl_2, 0.5 M, 2.0 eq.) was added. The reaction was stirred at room temperature for the time specified below, before it was quenched by the addition of $Na_2S_2O_5$ (12 eq.) in $NaHCO_3$ (aq., sat.) and extracted with CH_2Cl_2. The combined organic phases were washed with $NaHCO_3$ (aq., sat.) and brine, dried and concentrated to yield a crude oil, which was purified as specified below.

2-{4-[(2*S*)-1-*tert*-Butoxycarbonyl-pyrrolidine-2-yl)-carbonyl]-pyridin-2-yloxy}-acetamide (12a). The general procedure was applied to alcohol **11a** (55 mg, 0.16 mmol) for 1 hour. Purification by flash chromatography (EtOAc–heptane 4 : 1) afforded **12a** (33 mg, 61%) as a colorless oil: $[\alpha]_D$ −17.1 (*c* 0.7, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) (rotamers) δ 8.34 (d, 0.5H, J = 5.2 Hz), 8.30 (d, 0.5H, J = 5.2 Hz), 7.41–7.36 (m, 1H), 7.33 (s, 0.5H), 7.27 (s, 0.5H), 6.34 (br s, 1H), 5.88–5.65 (m, 1H), 5.17 (dd, 0.5H, J = 9.2, 3.9 Hz), 5.09–5.03 (m, 0.5H), 4.90–4.86 (m, 2H), 3.71–3.43 (m, 2H), 2.40–2.21 (m, 1H), 2.01–1.82 (m, 3H), 1.45 (s, 4.5H), 1.28 (s, 4.5H); ^{13}C NMR ($CDCl_3$) (two rotamers of equal intensity) δ 198.18, 197.94, 170.93, 170.76, 162.90, 162.80, 154.42, 153.48, 148.42, 148.21, 145.09, 144.88, 115.78, 115.54, 109.67, 109.32, 80.21, 80.09, 64.94, 64.86, 61.84, 61.38, 46.78, 46.58, 30.54, 29.44, 28.41, 28.21, 24.31, 23.51; IR 3337, 3057, 2977, 2882, 1681, 1557, 1410, 1163 cm^{-1}; HRMS (FAB) calcd for $C_{17}H_{24}N_3O_5$ [M + H]$^+$ 350.1716, found 350.1723. Byproduct **14**: ^{1}H NMR ($CDCl_3$) (rotamers) δ 7.45 (d, 0.55H, J = 6.7 Hz), 7.40 (d, 0.45H, J = 6.7 Hz), 7.07 (s, 0.45H), 7.04 (s, 0.55H), 6.75–6.67 (m, 1H), 5.12–5.06 (m, 0.45H), 5.03–4.97 (m, 0.55H), 3.70–3.40 (m, 3H), 2.38–2.23 (m, 1H), 1.99–1.53 (m, 3H), 1.45 (s, 4H), 1.31 (s, 5H); HRMS (FAB) calcd for $C_{15}H_{21}N_2O_4$ [M + H]$^+$ 293.1501, found 293.1504.

2-{3-Benzyl-4-[(2*S*)-1-*tert*-butoxycarbonyl-pyrrolidine-2-yl)-carbonyl]-pyridin-2-yloxy}-acetamide (12c). The general procedure was applied to alcohol **11c** (46 mg, 0.10 mmol) for three hours. Purification by flash chromatography (EtOAc–heptane 1 : 1)

afforded **12c** (39 mg, 86%) as white crystals: $[\alpha]_D$ +32 (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) (rotamers) δ 8.22–8.12 (m, 1H), 7.35–7.09 (m, 6H), 5.39–5.23 (m, 2H), 4.98–4.85 (m, 2H), 4.77–4.68 (m, 1H), 4.16–3.99 (m, 2H), 3.65–3.51 (m, 1H), 3.51–3.36 (m, 1H), 2.15–1.99 (m, 1H), 1.95–1.58 (m, 3H), 1.45 (s, 5H), 1.41 (s, 4H); ^{13}C NMR ($CDCl_3$) (two rotamers, major and minor) δ 203.17, 201.50, 171.10, 170.94, 160.84, 160.62, 154.48, 153.59, 148.04, 147.33, 145.33, 145.16, 139.79, 139.38, 128.69, 128.57, 128.33, 128.22, 126.59, 126.39, 120.82, 120.50, 115.81, 115.52, 80.36, 80.01, 64.37, 64.24, 64.01, 63.90, 46.81, 46.92, 32.30, 32.27, 29.12, 28.57, 28.38, 24.17, 22.64; HRMS (FAB) calcd for $C_{24}H_{29}N_3O_5$ $[M + H]^+$ 440.2185, found 440.2186.

General procedure for the Boc-deprotection reactions to produce 2, 3, 4, 5 and 6. The carbamate (1.0 eq.) was dissolved in CH_2Cl_2 ($\frac{3}{4}$ of the total volume) and trifluoroacetic acid ($\frac{1}{4}$ of the total volume) was added. The reaction was stirred for 5 to 8 minutes and then immediately concentrated under reduced pressure without heating to yield a crude solid, which was purified by reversed-phase HPLC to afford the products.

2-[4-((2*S*)-(Pyrrolidine-2-yl)-carbonyl)-pyridin-2-yloxy]-acetamide TFA-salt (2). The general procedure was applied to carbamate **12a** (15 mg, 0.043 mmol) in CH_2Cl_2–TFA (4 mL total volume) for 5 minutes. Freeze-drying allowed isolation of the TFA-salt of **2** (8.2 mg, 53%) as a white solid: $[\alpha]_D$ 0 (MeOH); ^1H NMR (CD_3OD) (keto : enol 1 : 1) δ 8.38 (d, 1H, $J = 5.3$ Hz), 8.20 (d, 1H, $J = 5.3$ Hz), 7.51 (d, 1H, $J = 5.3$ Hz), 7.46 (s, 1H), 7.10 (d, 1H, $J = 5.3$ Hz), 7.04 (s, 1H), 5.35 (dd, 1H, $J = 9.4$ and 6.7 Hz), 4.87 (s, 2H), 4.82 (s, 2H), 3.80–3.69 (m, 1H), 3.48–3.40 (m, 2H), 3.34–3.21 (m, 1H), 2.71–2.59 (m, 1H), 2.21–1.78 (m, 6H), 1.71–1.61 (m, 1H); ^{13}C NMR (CD_3OD) (keto and enol) δ 194.65, 164.99, 164.69, 152.36, 149.63, 148.68, 143.69, 117.14, 116.50, 111.67, 110.84, 99.29, 68.79, 65.41, 65.13, 64.79, 47.50, 47.16, 30.30, 26.42, 25.06, 25.02; HRMS (FAB) calcd for $C_{12}H_{16}N_3O_3$ $[M + H]^+$ 250.1192, found 250.1189.

2-[3-Benzyl-4-((2*S*)-(pyrrolidine-2-yl)-carbonyl)-pyridin-2-yloxy]-acetamide TFA-salt (4). The general procedure was applied to carbamate **12c** (10 mg, 0.023 mmol) in CH_2Cl_2–TFA (2 mL total volume) for 5 minutes. Freeze-drying allowed isolation of the TFA-salt of **4** (9 mg, 83%) as a white solid: $[\alpha]_D$ +12 (*c* 0.47, MeOH); ^1H NMR (CD_3OD) δ 8.30 (d, 1H, $J = 5.3$ Hz), 7.37 (d, 1H, $J = 5.3$ Hz), 7.32–7.24 (m, 2H), 7.22–7.17 (m, 3H), 5.28 (dd, 1H, $J = 9.4$, 7.2 Hz), 4.93–4.88 (in residual solvent peak, 2H), 4.34 (s, 2H), 3.40–3.33 (m, 1H), 3.31–3.22 (m, 1H), 2.27–2.16 (m, 1H), 2.01–1.89 (m, 1H), 1.75–1.63 (m, 1H), 1.47–1.37 (m, 1H); ^{13}C NMR (CD_3OD) δ 197.83, 173.74, 163.06, 146.95, 144.24, 141.10, 129.92, 129.53, 127.44, 124.62, 117.24, 66.21, 65.39, 47.34, 31.78, 28.70, 24.69; HRMS (FAB) calcd for $C_{19}H_{22}N_3O_3$ $[M + H]^+$ 340.1661, found 340.1664.

Pharmacology. Functional assays

The R-SAT (Receptor Selection and Amplification Technology™) assay was performed essentially as previously described.[24,26] Briefly, NIH/3T3 cells at 70–80% confluency were transfected, using Polyfect (Qiagen, Los Angeles, CA) as described in the manufacturer's protocols, with DNA encoding the human D2 receptor, Gqi5, and β-galactosidase, at 10 μg, 20 μg, and 50 μg per roller bottle, respectively. Cells were harvested 1 day after transfection and frozen at −135 °C. The cells were frozen by a standard method of slow freezing in a cryovial in a Styrofoam container placed at −70 °C. After overnight freezing at −70 °C, the cells were moved to a −135 °C cryogenic freezer for long-term storage. These freezers use a proprietary mix of refrigerants without liquid nitrogen. For each experiment testing potentiation of the NPA-induced response, cells were thawed, resuspended in media containing saturating amounts of *N*-propylapomorphine (1 μM), (NPA, pEC_{50} 7.6 ± 0.4, n = 7) and added directly to the compounds at a density of 20 000 cells per well in half-area 96-well plates. No difference was observed between cells transfected immediately prior to NPA activation[24] and cells frozen prior to use. After 5 days of incubation, the β-galactosidase activity was measured by the addition of *o*-nitrophenyl β-D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40 detergent). The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nM. For each experiment, compounds were plated in 12 wells for each concentration of compound tested. Each experiment was performed 3–10 times. Student's *t*-test was used to evaluate the statistical significance of the difference between the averaged values for treatment with both NPA and compound, and NPA alone. To test the agonist response at the D2 receptor, the compounds were plated in triplicate for each concentration of compound tested.

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