Constrained Analogues of Procaine as Novel Small Molecule Inhibitors of DNA Methyltransferase-1

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Constrained analogues of procaine were synthesized, and their inhibiting activity against DNMT1 was tested. Among them, the most potent compound, derivative **3b**, was also able to induce a recognizable demethylation of chromosomal satellite repeats in HL60 human myeloid leukemia cells and thus represents a lead compound for the development of a novel class of non-nucleoside DNMT1 inhibitors.

Introduction

Epigenetic alterations are increasingly recognized as valuable targets for the development of cancer therapies. They not only occur early in carcinogenesis but also are found in virtually all cases of cancer.[1–4] Importantly, epigenetic alterations do not involve changes in the DNA sequence and thus are potentially reversible.[2] Of the epigenetic changes seen in cancer, the most extensively studied is the increased methylation of CpG dinucleotides clustered in ~1 kb regions, termed CpG islands. This change in DNA methylation characteristically results in the transcriptional silencing of important cancer genes such as tumor suppressors and caretaker genes.[5]

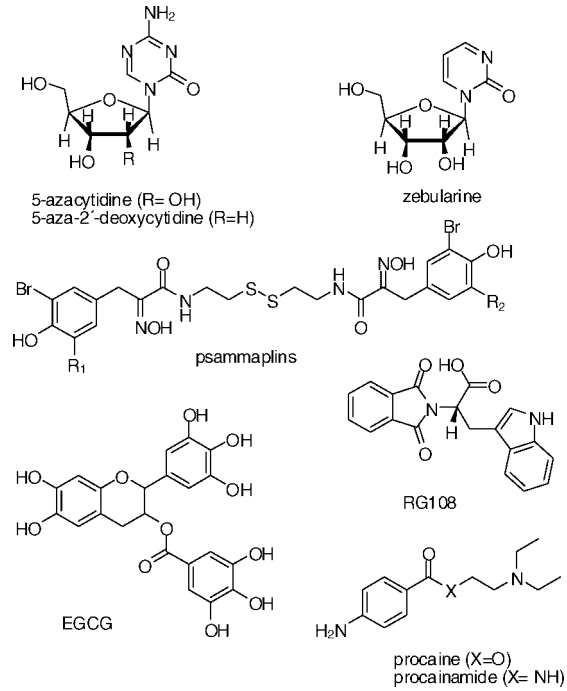
Nucleoside analogues like 5-azacytidine, 5-aza-2′-deoxycytidine (decitabine), or 2-pyrimidone-1-β-D-riboside (zebularine, Chart 1), though effective in inducing DNA demethylation and reactivation of hypermethylated genes, carry considerable concerns about toxicity,[6] which is probably associated with the drugs' incorporation into DNA. These concerns have strongly encouraged the search for non-nucleoside inhibitors of DNA methyltransferases (DNMTs[a]). However, only a limited number of them have been described so far (Chart 1), including dietary polyphenols like (−)-epigallocatechin-3-gallate (EGCG),[7] the bisulfide bromotyrosine derivatives psammaplins,[8] the L-tryptophan derivative RG108,[9,10] and the 4-aminobenzoic acid derivatives procaine and procainamide,[11–13] originally approved by the U.S. Food and Drug Administration as local anesthetic and for the treatment of cardiac arrhythmias, respectively.

Initially proposed as perturbative of the interactions between DNMTs and CpG-rich sequences, procainamide has been recently reported to specifically inhibit the maintenance methyltransferase activity of DNMT1 and to demethylate genes affected by promoter CpG island hypermethylation.[14]

Being interested in the development of small-molecule modulators of epigenetic targets,[9,10,15–20] we chose procaine as a lead structure for further modification. Because a high number of rotatable bonds is usually detrimental for druglikeness,[21,22] we decided to partially reduce the very high flexibility of procaine.

According to the frozen analogue approach,[23] we designed two series of molecules (**1–2** and **3–4**, Figure 1) where the *N*-alkylamide moiety of the lead scaffold was constrained into a 4-substituted or 5-substituted oxazoline ring. Herein, we report

Chart 1. Nucleoside and Non-Nucleoside Inhibitors of DNMTs



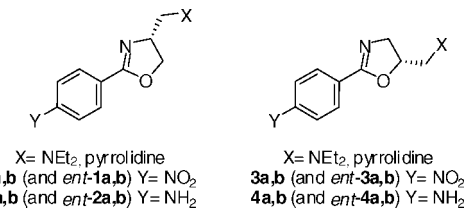


Figure 1. Restricted conformation analogues of procaine.

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[a] Abbreviations: DNMT, DNA methyltransferase; DNMT1, DNA methyltransferase-1; HL60, human myeloid leukemia cell line; SAM, *S*-adenosylmethionine; COBRA, combined bisulfite restriction analysis; C1S2, chromosome 1 satellite 2 repeats; TIMP3, tissue inhibitor of metalloproteinase 3; HCT116, human colon carcinoma cell line; RARβ2, retinoic acid receptor β2; MCF7, human breast adenocarcinoma cell line; Sf9, insect cell line derived from *Spodoptera frugiperda*; MOI, multiplicity of infection.

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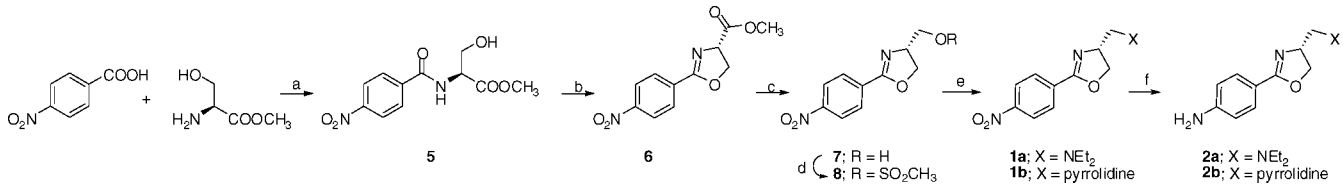
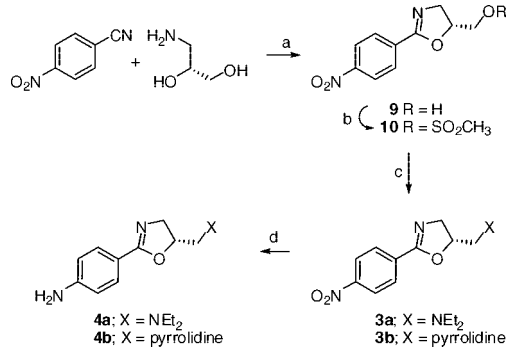
Scheme 1[a]



[a] Reagents and conditions: (a) NMM, HOBt, DCC, CH_2Cl_2/DMF 8:1, 0 °C to room temp; (b) Burgess reagent, THF, 80 °C; (c) $NaBH_4$, MeOH, 0 °C; (d) MsCl, TEA, THF, 0 °C to room temp; (e) diethylamine or pyrrolidine, DMF, 90 °C, sealed tube; (f) Zn, NH_4Cl, acetone/water 4:1, room temp.

Scheme 2[a]



[a] Reagents and conditions: (a) $ZnCl_2$, chlorobenzene/NMP 95:5, reflux; (b) MsCl, TEA, THF, 0 °C to room temp; (c) diethylamine or pyrrolidine, DMF, 90 °C, sealed tube; (d) Zn, NH_4Cl, acetone/water 4:1, room temp.

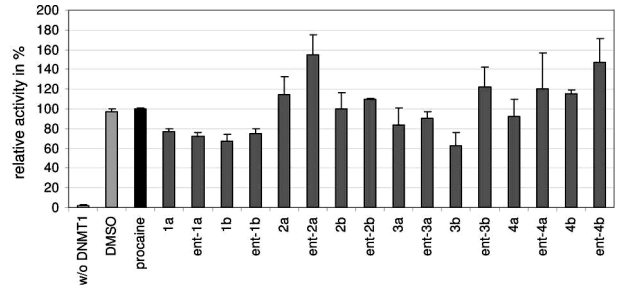


Figure 2. In vitro methylation assay for **1**–**4** against recombinant human DNMT1. Procaine was used as reference drug. All compounds were tested as 2 mM DMSO solutions. Error bars indicate standard deviations of each measurement. DMSO (2 mM) alone did not affect the enzymatic activity of DNMT1. Data are reported as the mean ± SD of three independent experiments.

the synthesis of such compounds and their inhibitory activity toward DNA methyltransferases in vitro and in vivo.

Chemistry

The preparation of derivatives **1a**,**b** and **2a**,**b** is reported in Scheme 1. Peptide coupling of 4-nitrobenzoic acid with (*S*)-serine methyl ester hydrochloride gave carboxamide **5**, which underwent a smooth cyclization reaction with Burgess reagent[24] to give the oxazoline derivative **6** that was then quantitatively reduced to the primary alcohol **7**.[25] The transformation of the latter into the amine **1a**,**b** was performed by mesylation and subsequent substitution reaction with diethylamine or pyrrolidine, respectively. Finally, reduction of nitro group using zinc dust and NH_4Cl in ethanol[26] yielded **2a**,**b** as hydrochloride salts (Scheme 1).

The enantiomers *ent*-**1a**,**b** and *ent*-**2a**,**b** were prepared analogously starting from (*R*)-serine methyl ester.

Derivatives **3a**,**b** and **4a**,**b** were obtained by a slight modification of the general stereospecifical route to 2-oxazolines previously reported by Serrano et al. (Scheme 2).[27] Briefly, a chlorobenzene solution of 4-nitrobenzonitrile and (*R*)-3-aminopropane-1,2-diol was refluxed in the presence of a catalytic amount of zinc chloride and 1-methylpyrrolidin-2-one as cosolvent to afford the alcohol **9** in 63% yield. The nucleophilic displacement of the corresponding mesylate **10** with diethylamine and pyrrolidine furnished **3a** and **3b**, respectively, which were finally reduced with zinc and NH_4Cl in ethanol to give **4a** and **4b** as hydrochloride salts (Scheme 2).

The enantiomers *ent*-**3a**,**b** and *ent*-**4a**,**b** were prepared analogously starting from (*S*)-3-aminopropane-1,2-diol.

Results and Discussion

In order to establish a biochemical assay for testing the activity of derivatives **1**–**4** against DNMT1, we generated recombinant baculoviruses expressing human DNMT1 with an N-terminal epitope tag. Sf9 insect cells were infected with a MOI of 5 for 48 h and DNMT1 was purified from cleared lysates of infected cells by Ni-NTA agarose affinity chromatography (Supporting Information). The identity and integrity of recombinant DNMT1 were confirmed by SDS gel electrophoresis and Western blotting with a DNMT1 specific antibody (data not shown). In order to determine the enzymatic activity of purified DNMT1, we established a biochemical DNA methylation assay. Methylation rates were measured by the incorporation of radioactive labeled methyl groups into oligonucleotide substrates. Tritium labeled *S*-adenosylmethionine (SAM) was used as methyl group donor. The entire reaction was spotted manually onto cellulose membranes, washed several times, dried, and after addition of scintillation cocktail, subjected to a scintillation counting. The results showed that the recombinant DNMT1 enzyme has substantial enzymatic activity (Figure 2). In subsequent experiments, this assay was used to test the activity of derivatives **1**–**4** (2 mM DMSO solutions). Procaine and *S*-adenosylhomocysteine (data not shown) were used as references (2 mM DMSO solutions).

Similar to what was previously reported,[28] procaine did not show any activity in this assay. Yet derivatives **1** and **3** exhibited a weak but distinct inhibition of DNMT1 (Figure 2). In particular, it is noteworthy that this activity is related to the presence of the nitro group on the benzene ring (compounds **1a**,**b**, **3a**,**b**, and corresponding enantiomers[29]), whereas their anilino counterparts (**2a**,**b**, **4a**,**b**, and corresponding enantiomers) were consistently less active. The replacement of the diethylamino function with a pyrrolidine ring further improved the inhibitory efficacy of tested compounds (for example, compare the inhibition of **1a** and **3a** with the inhibition of their pyrrolidino counterparts **1b** and **3b**, respectively). Remarkably, this was particularly evident in nitro derivatives frozen in the 5-substituted oxazoline conformation. In fact, the gain in activity is more marked between **3a** and **3b** (5-substituted oxazolino derivatives) than between **1a** and **1b** (4-substituted equivalents). Finally, it is also worth mentioning that the inhibitory potencies of **1**–**4** were always higher than those displayed by their enantiomers

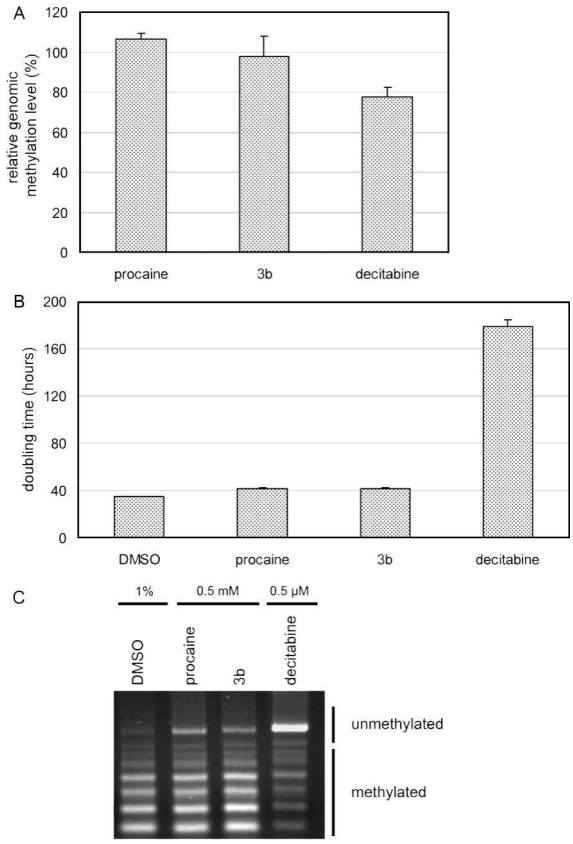


Figure 3. Cellular methylation assays. Human myeloid leukemia (HL60) cells were incubated for 72 h with DMSO (1%), procaine (0.5 mM), decitabine (0.5 μM), and test compound **3b** (0.5 mM). (A) Genomic cytosine methylation levels were determined by capillary electrophoretic analysis of isolated genomic DNA as described in Supporting Information. Methylation levels are shown relative to DMSO controls. Decitabine treatment induced a strong, **3b** a weaker, genomic demethylation. Data are reported as the mean ± SD of three independent experiments. (B) Proliferation assay. HL60 cells were seeded in duplicate in six-well plates, incubated for 72 h, and counted for the determination of doubling times. (C) COBRA assay of chromosomal satellite repeats (C1S2) as described in Supporting Information. DMSO treated cells revealed methylated satellites, but procaine as well as **3b** induced minor demethylation of the repetitive elements. Decitabine treatment was included as positive control and showed the strongest demethylating effect.

ent-**1**–**4**, with the sole exception of **1a**, which was slightly less active than *ent*-**1a**.

As **3b** showed the strongest inhibitory effects among tested derivatives, we also explored its effect on the genomic DNA methylation level of HL60 human myeloid leukemia cells. Cells were incubated for 72 h with 0.5 mM drug solutions in DMSO, and genomic cytosine methylation levels were quantitatively determined by capillary electrophoresis.[30] The proliferation of HL60 cells (population doubling time) was also assessed.

The results demonstrated a slight decrease of the global methylation level after **3b** treatment compared to procaine (Figure 3A). More pronounced demethylation was observed with 5-aza-2′-deoxycytidine (Figure 3A), but this effect was accompanied by substantial cytotoxicity (Figure 3B). However, **3b** did not reveal any detectable cytotoxic effects on HL60 cells (Figure 3B), thus encouraging the further development of this scaffold. We also used combined bisulfite restriction analysis (COBRA)[31] to analyze the methylation status of chromosomal satellite repeats. This assay has been demonstrated to be particularly sensitive toward demethylation induced by DNMT inhibitors.[32] A weak but detectable demethylation of these sequences was observed for both procaine and **3b** (Figure 3C), which is consistent with the previously reported slight local demethylation of the TIMP3 locus in HCT116[14] or RARβ2 locus in MCF7 cells,[12] after treatment with procainamide or procaine, respectively.

Conclusions

In conclusion, we prepared a series of constrained analogues of procaine and tested their inhibiting activity against DNMT1. Among them, derivative **3b** was proven the most interesting compound, exhibiting the highest inhibiting potency, and when tested for its effects on the genome methylation levels in HL60 human myeloid leukemia cells, it revealed a recognizable demethylation of chromosomal satellite repeats. These effects are cell type specific (data not shown), and though weaker than those obtained with azanucleosides, they are fairly comparable to other non-nucleoside inhibitors. Importantly, the high cytotoxicity and the low therapeutic index of nucleoside analogues make the development of novel non-nucleoside inhibitors highly desirable. Thus, derivative **3b** might be considered a lead compound for further studies in this field. Extensive SAR analysis and molecular modeling studies are ongoing to increase the knowledge within these series of DNMT1 inhibitors.

Experimental Section

Preparation of (*S*)-Methyl 2-(4-Nitrophenyl)-4,5-dihydrooxazole-4-carboxylate (6) and (*R*)-Methyl 2-(4-Nitrophenyl)-4,5-dihydrooxazole-4-carboxylate (*ent*-6). (*S*)-*N*-(4-Nitrobenzoyl)-serine methyl ester (**5**, 1000.0 mg, 3.728 mmol) was dissolved in dry THF (14 mL) in a sealed tube, and Burgess reagent [(methoxycarbonylsulfamoyl)triethylammonium hydroxide inner salt] (1066.2 mg, 4.474 mmol) was added. After the mixture was heated at 80 °C for 1 h, the solution was evaporated and the crude purified by column chromatography on silica gel (gradient, hexane/EtOAc, 8:2 to 4:6) to afford pure **6** (727.6 mg, 78%) as a white solid (mp 77–78 °C). (*R*)-Methyl 2-(4-nitrophenyl)-4,5-dihydrooxazole-4-carboxylate (*ent*-**6**) was prepared under the same reaction conditions, starting from (*R*)-*N*-(4-nitrobenzoyl)serine methyl ester (*ent*-**5**). **6**: $[\alpha]^{23}_{D}$ +111.9° (*c* 3.68, AcOEt). *ent*-**6**: $[\alpha]^{23}_{D}$ –110.8° (*c* 3.92, AcOEt). ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H), 4.66 (dd, 1H, J = 10.7, 8.8 Hz), 4.76 (dd, 1H, J = 8.8, 8.1 Hz), 5.00 (dd, 1H, J = 10.7, 8.1 Hz), 8.15 (d, 2H, J = 9.0 Hz), 8.27 (d, 2H, J = 9.0 Hz). ESI-MS *m*/*z*: 251 $(M + H)^+$. Anal. ($C_{11}H_{10}N_2O_5$) C, H, N.

Preparation of (*R*)-[2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl]methanol (7) and (*S*)-[2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl]methanol (*ent*-7). To a solution of (*S*)-2-(4-nitrophenyl)-4,5-dihydrooxazole-4-carboxylic acid methyl ester (**6**, 700.0 mg, 2.798 mmol) in MeOH (100 mL) cooled at 0 °C, $NaBH_4$ was added until disappearance of the starting material (TLC: silica gel, hexane/EtOAc 3:7). The reaction mixture was evaporated, taken up with water (100 mL), and extracted with chloroform (3 × 60 mL). The organic phase was dried and the solvent evaporated to give pure **7** (608.7 mg, 98%) as a white solid (mp 133–134 °C). (*S*)-[2-(4-nitrophenyl)-4,5-dihydrooxazol-4-yl]-methanol (*ent*-**7**) was prepared under the same reaction conditions, starting from (*R*)-(4-nitrophenyl)-4,5-dihydrooxazole-4-carboxylic acid methyl ester (*ent*-**6**). **7**: $[\alpha]^{23}_{D}$ +61.55° (*c* 1.06, $CHCl_3$). *ent*-**7**: $[\alpha]^{23}_{D}$ –62.85° (*c* 1.07, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.94 (br s, 1H), 3.72 (dd, 1H, J = 11.4, 4.1 Hz), 3.98 (dd, 1H, J = 11.4, 3.5 Hz), 4.40–4.60 (m, 3H), 7.93 (d, 2H, J = 8.9 Hz), 8.12 (d, 2H, J = 8.9 Hz). ESI-MS *m*/*z*: 223 $(M + H)^+$.

Preparation of (*S*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl Methanesulfonate (8) and (*R*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl Methanesulfonate (*ent*-8). To a solution of (*R*)-[2-(4-nitrophenyl)-4,5-dihydrooxazol-4-yl]methanol (**7**, 370.0 mg, 1.66 mmol) and triethylamine (0.278 mL, 2.00 mmol)

in dry THF (20 mL) methanesulfonyl chloride (0.155 mL, 2.00 mmol) was added at 0 °C. The resulting mixture was stirred at room temperature for 45 min, and then the solvent was evaporated. The residue was taken up with water (20 mL) and extracted with chloroform (3 × 40 mL). The organic phase was dried and the solvent evaporated to give mesylate **8** (502.5 mg, 98%), which was used without further purification in the subsequent reaction. (*R*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl methanesulfonate (*ent*-**8**) was prepared under the same reaction conditions, starting from *ent*-**7**.

General Procedure for the Synthesis of (2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methanamines 1a,b and *ent*-1a,b. Example: (*R*)-*N*-Ethyl-*N*-((2-(4-nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl)ethanamine (1a). (*S*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl methanesulfonate (**8**, 342.3 mg, 1.14 mmol) was dissolved in dry DMF (14 mL) in a sealed tube, and diethylamine (1.50 mL, 34.2 mmol) was added. After being heated at 90 °C overnight, the mixture was evaporated, taken up with water (20 mL), and extracted with ethyl acetate (3 × 50 mL). The organic layer was dried and evaporated. The crude was purified by column chromatography on silica gel (gradient, chloroform/methanol, 98:2 to 90:10) to afford pure **1a** (253.8 mg, 90%) as a yellow solid (mp 79–80 °C). $[\alpha]^{23}_{D}$ +21.05° (*c* 3.80, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.02 (t, 6H, J = 7.1), 2.46–2.70 (m, 5H), 2.82 (dd, 1H, J = 12.7, 4.5 Hz), 4.26–4.32 (m, 1H), 4.45–4.53 (m, 2H), 8.11 (d, 2H, J = 8.9 Hz), 8.23 (d, 2H, J = 8.9 Hz). ESI-MS *m*/*z*: 278 $(M + H)^+$. Anal. ($C_{14}H_{19}N_3O_3$) C, H, N. (2-(4-Nitrophenyl)-4,5-dihydrooxazol-4-yl)methanamines **1b** and ent-**1a**,**b** were prepared under the same reaction conditions, starting from mesylates **8** or *ent*-**8** and the proper amine.

General Procedure for the Synthesis of 4-Substituted-4-(4,5-dihydrooxazol-2-yl)anilines 2a,b and *ent*-2a,b. Example: (*R*)-4-(4-(Diethylaminomethyl)-4,5-dihydrooxazol-2-yl)aniline (2a). A solution of (*R*)-*N*-ethyl-*N*-((2-(4-nitrophenyl)-4,5-dihydrooxazol-4-yl)methyl)ethanamine (**1a**, 250.0 mg, 0.9 mmol) in acetone/water 4:1 (10 mL) was treated with zinc dust (471.5 mg, 7.2 mmol) and ammonium chloride (385.5 mg, 7.2 mmol), and the resulting suspension was stirred for 45 min at 20 °C. The reaction mixture was then filtered, and the filtrate was evaporated. The crude was purified by column chromatography on basic alumina, eluting with chloroform to afford the pure hydrochloride of **2a** (250.0 mg, 97%) as a hygroscopic yellow solid foam. $[\alpha]^{23}_{D}$ +1.62° (*c* 3.08, MeOH). ^{1}H NMR (DMSO-d_6): δ 0.94 (t, 6H, J = 7.0) 2.29–2.65 (m, 6H), 4.00–4.05 (m, 1H), 4.21–4.39 (m, 2H), 5.66 (s, 2H), 6.54 (d, 2H, J = 8.3 Hz), 7.51 (d, 2H, J = 8.3 Hz). ESI-MS *m*/*z*: 248 $(M + H)^+$. Anal. ($C_{14}H_{21}N_3O\cdot HCl$) C, H, N. 4-(4,5-Dihydrooxazol-2-yl)anilines **2b** and *ent*-**2a**,**b** were prepared under the same reaction conditions, starting from the corresponding nitro derivative **1b** or *ent*-**1a**,**b**.

Preparation of (*R*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-5-yl)methanol (9) and (*S*)-(2-(4-Nitrophenyl)-4,5-dihydrooxazol-5-yl)methanol (*ent*-9). Zinc chloride (460.0 mg, 50 mol %) was melted under vacuum in a two-necked flask, and chlorobenzene (15 mL) was added under nitrogen at room temperature, followed by 4-nitrobenzonitrile (1000 mg, 6.75 mmol), (*R*)-3-aminopropane-1,2-diol (738 mg, 8.10 mmol), and 1-methyl-2-pyrrolidinone (0.5 mL). The resulting yellow solution was heated at reflux for 24 h. The solvent was then removed under reduced pressure and the oily residue dissolved in dichloromethane (30 mL). The solution was washed with saturated aqueous sodium hydrogen carbonate (2 × 10 mL), dried, and concentrated under vacuum. The crude was purified by column chromatography on silica gel (gradient, chloroform/methanol, 98:2 to 90:10) to afford pure **9** (944.8 mg, 63%) as a yellow solid (mp 129–131 °C). *ent*-**9** was prepared under the same reaction conditions, starting from (*S*)-3-aminopropane-1,2-diol. **9**: $[\alpha]^{23}_{D}$ +5.39° (*c* 5.560, $CHCl_3$). *ent*-**9**: $[\alpha]^{23}_{D}$ –5.77° (*c* 5.195, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.65 (br s, 1H), 3.75 (dd, 1H, J = 12.3, 5.4 Hz), 3.82–3.95 (m, 2H), 4.12 (dd, 1H, J = 15.2, 10.0 Hz), 4.82–4.90 (m, 1H), 8.06 (d, 2H, J = 9.0 Hz), 8.20 (d, 2H, J = 9.0 Hz). ESI-MS *m*/*z*: 223 $(M + H)^+$. Anal. ($C_{10}H_{10}N_2O_4$) C, H, N.

The (2-(4-nitrophenyl)-4,5-dihydrooxazol-5-yl)methanamines 3a,b and *ent*-3a,b were prepared via the mesylates **10** according to the same procedure described above for **1a**.

The 5-substituted-4-(4,5-dihydrooxazol-2-yl)anilines 4a,b and *ent*-4a,b were prepared by reduction of the corresponding nitro derivatives **3a**,**b** or *ent*-**3a**,**b** according to the same procedure described above for **2a**.

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Supporting Information Available: Experimental chemical and biological procedures and characterization data for **1**−**4** and for intermediates **5**–**10**, as well as for the corresponding enantiomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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