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Bioorganic & Medicinal Chemistry 14 (2006) 4393–4401
Bioorganic & Medicinal Chemistry

Synthesis of enantiopure Δ^2-isoxazoline derivatives and evaluation of their affinity and efficacy profiles at human β-adrenergic receptor subtypes

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Received 11 January 2006; revised 15 February 2006; accepted 21 February 2006
Available online 10 March 2006

Abstract—The new enantiomerically pure 3-substituted-Δ^2-isoxazolin-5-yl-ethanolamines (+)-**6a**/(−)-**6b**, (−)-**6a**/(+)-**6b**, and (+)-**7a**/(−)-**7b**, prepared via a 1,3-dipolar cycloaddition-based approach, were tested for their affinity at human β_1-, β_2-, and β_3-adrenergic receptor (β-AR) subtypes stably expressed in CHO cells. The corresponding 3-isopropenyl derivatives (+)-**5a**/(−)-**5b**, (−)-**5a**/(+)-**5b**, and some isoxazole analogs were also tested. The binding affinities at the β-ARs of the isoxazolinyl amino alcohols were significantly lower than those of the corresponding isoxazole derivatives. A stereochemical effect was observed, since the process of molecular recognition is predominantly controlled by the (*S*)-configuration of the stereogenic center located at the 5 position of the heterocycle rather than by that of the stereocenter carrying the secondary alcohol group. On the contrary, the stereochemical features marginally affected the efficacy response; as a matter of fact, functional tests carried out on Δ^2-isoxazoline derivatives provided with a detectable binding affinity showed the overall profile of neutral antagonists at all three β-AR subtypes.

1. Introduction

β-Adrenergic receptors (β-ARs) belong to the superfamily of rhodopsin-like G protein-coupled receptors (GPCRs) mediating the physiological responses to noradrenaline (NA) and adrenaline (A). At present, three β-AR subtypes, classified as β_1-, β_2-, and β_3-adrenergic receptors, have been identified by means of pharmacological investigations as well as through cloning of the receptors. Functionally significant levels of β_1-ARs are found in the heart, kidney, and brain,[1] β_2-ARs are predominant on vascular, uterine, and airway smooth muscle,[2] and β_3-ARs are mainly expressed in adipose tissue.[3] All of them couple primarily to $G\alpha_s$ to stimulate adenylyl cyclase, even though they can also couple to $G\alpha_i$ in some cells under certain conditions.[4]

A number of agonists and antagonists for β-ARs have found application for the clinical treatment of cardiovascular diseases and asthma. In particular, β_1-antagonists ('beta blockers') are routinely prescribed in hypertension, coronary heart disease (CHD), and heart failure, whereas β_2-AR agonists are commonly used in the treatment of asthma owing to their regulatory effect on bronchial tone. Moreover, research programs have been addressing the development of selective β_3-AR agonists or partial agonists as potential drugs for the treatment of obesity and diabetes.[5] However, the structural requirements for a subtype-selective receptor interaction of both agonists and antagonists have not been completely elucidated yet. This is in part due to the limited comparability of pharmacological data from quite different biological model systems resulting in a poor correlation between the pharmacology of various compounds and their therapeutic effectiveness in animal models.

In the past, we synthesized and tested a set of 3-substituted Δ^2-isoxazolin-5-yl- and isoxazol-5-yl-ethanolamines as hybrid compounds containing structural elements common to the nonselective β_1/β_2 antagonist

Keywords: Synthesis; Δ^2-Isoxazoline derivatives; Human β-adrenergic receptor subtypes; Binding affinity; Efficacy; Antagonist.
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doi:10.1016/j.bmc.2006.02.038

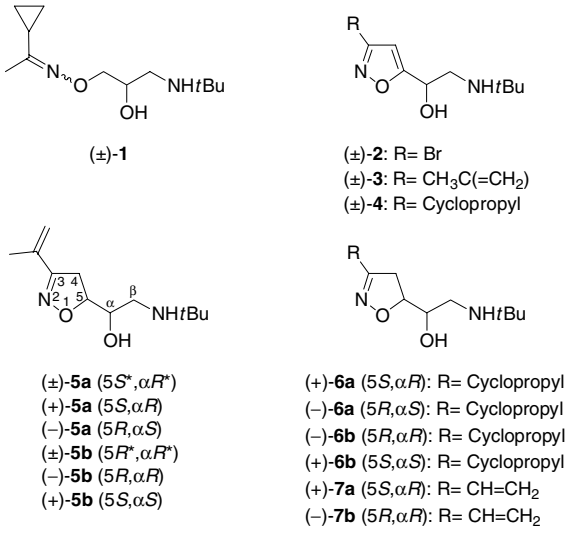


Figure 1. Structure of the model and designed compounds.

Falintolol (±)-**1**,[6,7] an aliphatic oxime ether, and to Broxaterol (±)-**2**, a β_2-selective agonist initially developed as a potential bronchodilatory agent in the therapy of asthma,[8–11] which was later on discontinued (Fig. 1).

Among the investigated compounds, isoxazoles (±)-**3** and (±)-**4** behaved as partial agonists at β_1-AR and as antagonists at β_2-AR when tested in isolated guinea pig atria and trachea tissue preparations, respectively.[12] In a parallel study, isoxazoline (±)-**5a** (Fig. 1) displayed an affinity profile comparable to those of both (±)-**1** and (±)-**2** when assayed at rat C6 glioma cells (β_1-receptors) and at Chinese hamster ovary (CHO) cells transfected with human β_2-AR.[13] In addition, derivative (±)-**5a** showed the pharmacological profile of an antagonist at both β_1- and β_2-ARs, and, consequently, appeared to resemble Falintolol or related linear oxime ethers rather than Broxaterol.[13]

Since some of us recently generated CHO cells stably expressing the three human β-ARs at comparable levels,[14] we planned to investigate the affinity/efficacy profile at the three receptors of Δ^2-isoxazolines **5**–**7** (Fig. 1). In this paper, we report the synthesis of the new stereoisomeric pairs of 3-cyclopropyl (+)-**6a**/(−)-**6b**, (−)-**6a**/(+)-**6b** and 3-vinyl (+)-**7a**/(−)-**7b** derivatives together with their binding affinity; the binding affinity of the related analogs reported in Figure 1 has also been evaluated. Derivatives with significant binding affinity were also tested in functional assays at the human β_1-, β_2-, and β_3-receptor subtypes.

2. Chemistry

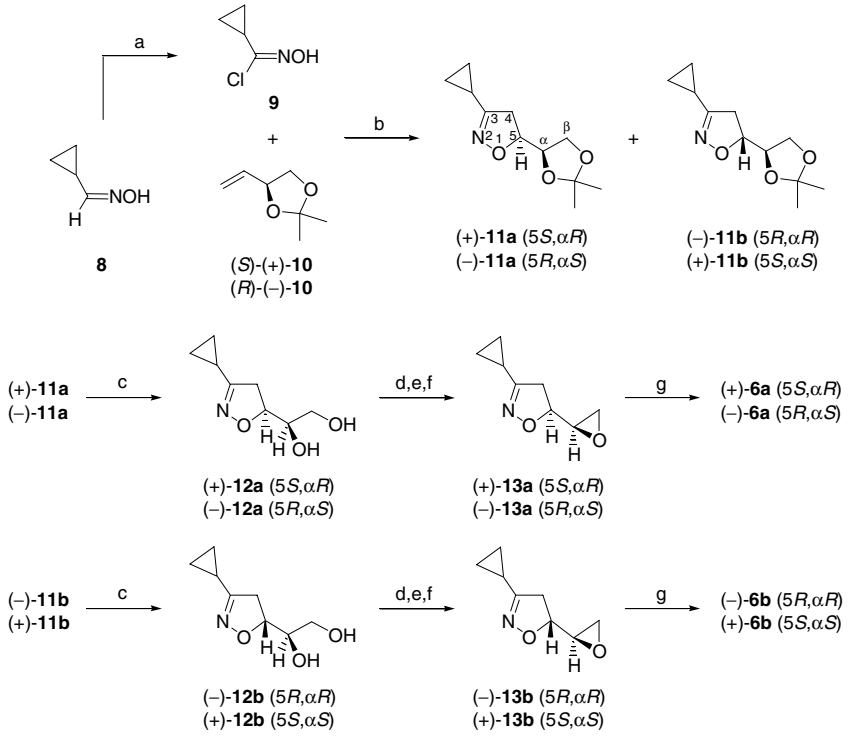
The novel target 3-cyclopropyl-Δ^2-isoxazolin-5-yl-ethanolamines were prepared following a strategy similar to that applied to the synthesis of aminoalcohols (+)-**5a**/(−)-**5a** and (−)-**5b**/(+)-**5b**.[15] The key step of this approach is represented by the 1,3-dipolar cycloaddition of cyclopropancarbonitrile oxide, generated in situ by treating cyclopropancarbohydroximoyl chloride **9** with a base, to alkenes (*S*)-(+)-**10**[16,17] and (*R*)-(−)-**10**[17,18] (Scheme 1). The stable precursor of the 1,3-dipole was obtained by chlorination of cyclopropanecarboxaldehyde oxime **8**[12] with benzyltrimethylammonium tetrachloroiodate (BTMA ICl_4), according to a published protocol.[19]

As previously reported, cycloaddition of various nitrile oxides to **10** is characterized by a pronounced *anti* diastereoselectivity.[20–22] Indeed, the reaction of (*S*)-(+)-**10** [(*R*)-(−)-**10**] with cyclopropancarbonitrile oxide produced *anti* (+)-**11a** [(−)-**11a**] and *syn* (−)-**11b** [(+)-**11b**] cycloadducts in a 79:21 ratio. After a column chromatography separation, the two diastereomers were submitted to a series of transformations involving substituents at positions 3 and 5 of the heterocylic ring. Thus, the Amberlite IR-120 promoted acetonide cleavage provided the related diols (+)-**12a** [(−)-**12a**] and (−)-**12b** [(+)-**12b**], which smoothly underwent a Sharpless' 'one-pot' procedure[23] affording epoxides (+)-**13a** [(−)-**13a**] and (−)-**13b** [(+)-**13b**] with retention of configuration at C-α. Treatment of (+)-**13a** [(−)-**13a**] and (−)-**13b** [(+)-**13b**] with an excess of *tert*-butylamine in refluxing methanol gave the desired *anti* (5*S*,α*R*)-(+)-**6a** [(5*R*,α*S*)-(−)-**6a**] and *syn* (5*R*,α*R*)-(−)-**6b** [(5*S*,α*S*)-(+)-**6b**] ethanolamines (Scheme 1).

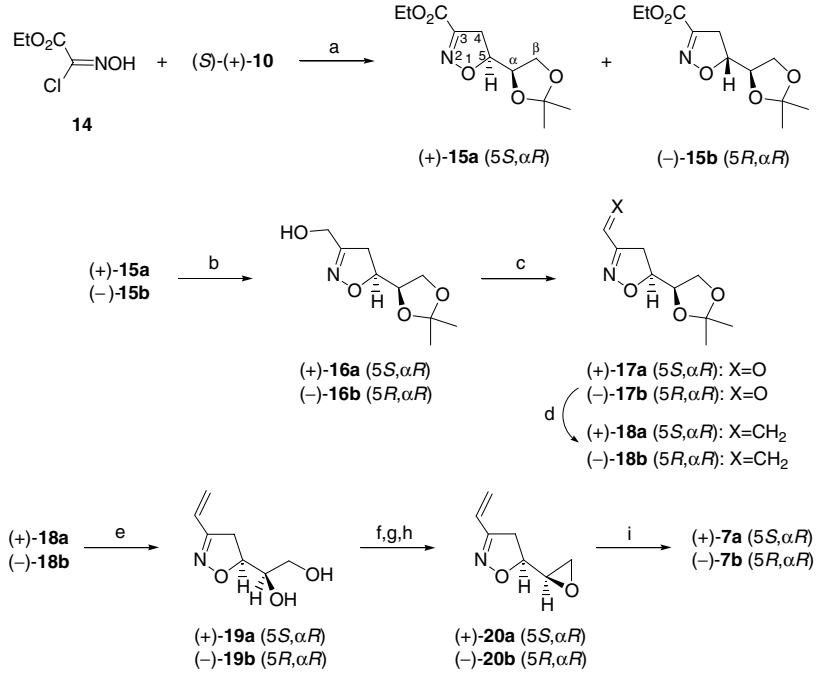
Quite similarly, cycloaddition of ethoxycarbonylformonitrile oxide, liberated in situ from ethyl 2-chloro-2-(hydroxyimino)-acetate **14**, to (*S*)-(+)-**10** produced Δ^2-isoxazolines (+)-**15a** and (−)-**15b** in a 72:28 ratio (Scheme 2).[20,22] The two stereoisomeric esters were separately functionalized at position 3 through a reduction with sodium borohydride[24] to give primary alcohols (+)-**16a** and (−)-**16b**, followed by oxidation to the related aldehydes (+)-**17a** and (−)-**17b**, and then by a Wittig olefination which afforded 3-vinyl-Δ^2-isoxazolines (+)-**18a** and (−)-**18b**. According to the previously discussed transformations of the side chain located at position 5, acetonides (+)-**18a** and (−)-**18b** were converted into diols (+)-**19a** and (−)-**19b**, then into epoxides (+)-**20a** and (−)-**20b**, which were subsequently transformed into final aminoalcohols (5*S*,α*R*)-(+)-**7a** and (5*R*,α*R*)-(−)-**7b**, respectively (Scheme 2). Absolute configurations to individual stereoisomeric aminoalcohols **6a**–**b** and **7a**–**b** were assigned following the methodology previously applied to their analogs **5a**–**b**.[15]

3. Results and discussion

The 1:1 oxalates of Δ^2-isoxazolinyl-ethanolamines (+)-**5a**/(−)-**5a**, (−)-**5b**/(+)-**5b**, (+)-**6a**/(−)-**6a**, (−)-**6b**/(+)-**6b**, and (+)-**7a**/(−)-**7b** were tested for binding affinity at human β_1-, β_2-, and β_3-ARs in membranes from CHO cells stably transfected with the respective receptor subtypes.[14] Table 1 reports the dissociation constants (K_i values) of the investigated compounds from competition experiments with [^{125}I]cyanopindolol ([^{125}I]CYP) as the radioligand. The corresponding K_i values for (±)-Falintolol oxalate **1**,[6] (±)-Broxaterol **2**[11], and the two isoxazoles (±)-**3**[12] and (±)-**4**[12] have been included for



Scheme 1. Reagents: (a) BTMA ICl_4/CH_2Cl_2; (b) AcOEt/$NaHCO_3$; (c) Amberlite IR-120/MeOH; (d) $MeC(OMe)_3$/*p*-TosOH; (e) Me_3SiCl/CH_2Cl_2; (f) K_2CO_3/MeOH; (g) *t*-$BuNH_2$/MeOH.



Scheme 2. Reagents: (a) AcOEt/$NaHCO_3$; (b) $NaBH_4$/EtOH; (c) PCC/AcONa/CH_2Cl_2; (d) $Ph_3P{=}CH_2$; (e) Amberlite IR-120/MeOH; (f) $MeC(OMe)_3$/*p*-TosOH; (g) Me_3SiCl/CH_2Cl_2; (h) K_2CO_3/MeOH; (i) *t*-$BuNH_2$/MeOH.

comparison. Since binding experiments were carried out in the presence of 100 μM GTP, all K_i values for agonists reflect low affinity binding.

Within the group of Δ^2-isoxazoline derivatives under study, the *anti* 3-isopropenyl (5*S*,α*R*)-(+)-**5a** isomer exhibited the highest affinity for β_1-ARs (K_i = 433 nM) and β_2ARs (K_i = 190 nM), but bound with lower affinity to β_3-ARs (K_i = 5350 nM). However, at the β_3-subtype (+)-**5a** represented the only isoxazolinyl ethanolamine derivative with a detectable affinity, since all the related analogs **5**–**7** had K_i values higher than 100,000 nM.

Table 1. Binding affinities from competition experiments for the ligands under study at human β-adrenergic receptor subtypes

Compound	β_1-receptors		β_2-receptors		β_3-receptors	
	K_i (nM)	95% confidence limits	K_i (nM)	95% confidence limits	K_i (nM)	95% confidence limits
(+)-**5a**	433	300–625	190	130–270	5350	5100–5610
(−)-**5a**	16,500	11,400–24,000	2260	1760–2910	$>10^5$	—
(+)-**5b**	5370	3690–7820	1620	1180–2220	$>10^5$	—
(−)-**5b**	$>10^5$	—	$>10^5$	—	$>10^5$	—
(+)-**6a**	15,300	10,900–21,600	1,290	840–2000	$>10^5$	—
(−)-**6a**	$>10^5$	—	33,200	18,500–59,600	$>10^5$	—
(+)-**6b**	$>10^5$	—	32,800	21,300–50,400	$>10^5$	—
(−)-**6b**	$>10^5$	—	$>10^5$	—	$>10^5$	—
(+)-**7a**	10,800	9000–13,000	1910	1660–2200	$>10^5$	—
(−)-**7b**	$>10^5$	—	$>10^5$		$>10^5$	—
(±)-**1**	53.5	39.1–73.2	27.9	20.6–37.7	5120	3530–7420
(±)-**2**	1310	930–1860	1290	916–1810	3990	3470–4590
(±)-**3**	54.1	35.7–82.2	18.0	11.3–28.5	1965	1590–2430
(±)-**4**	1000	682–1470	418	375–466	24,680	10,400–58,800

50–80 pM [^{125}I]CYP were used as radioligand. Experiments were done in the presence of 100 μM GTP. K_i values were calculated with the program SCTFIT and represent geometric mean values of at least three different experiments done in triplicate.

The 3-isopropenyl-isoxazole (±)-**3**, whose K_i values are quite comparable with those of Falintolol (±)-**1**, showed pharmacological characteristics which paralleled those of the structural analog (+)-**5a**. However, the affinity values of (±)-**3** at all β-AR subtypes (β_1: K_i = 54.1 nM; β_2: K_i = 18.0 nM; β_3: K_i = 1.965 nM) were roughly one order of magnitude higher than those of (+)-**5a**. Therefore, replacement of the isoxazole nucleus with the Δ^2-isoxazoline ring brings about a reduction in the binding affinity. A parallel behavior in the affinity profile was revealed by comparing the K_i values of the 3-cyclopropyl-isoxazole (±)-**4** with those of its related *anti* isoxazoline aminoalcohol (5*S*,α*R*)-(+)-**6a**. Although the most potent isoxazolinyl ethanolamines under investigation show only moderate affinities mainly at the β_1- and β_2-ARs, these compounds provide a valuable tool for further studies on the process of molecular recognition of the ligands by the three receptor subtypes, since the binding affinity of the stereoisomers varies dramatically. The effect of the stereochemistry on affinity is particularly visible at the β_1- and β_2-subtypes, and shows that ligand recognition appears to be mainly dictated by the stereogenic center at C-5. This conclusion is supported by considering the binding data of the three *anti*/*syn* stereoisomeric pairs (5*S*,α*R*)-**5a**/(5*R*,α*R*)-**5b**, (5*S*,α*R*)-**6a**/(5*R*,α*R*)-**6b**, and (5*S*,α*R*)-**7a**/(5*R*,α*R*)-**7b**, and those of the *syn*/*anti* stereoisomers (5*S*,α*S*)-**5b**/(5*R*,α*S*)-**5a**. Thus, the ligand–receptor interaction is predominantly controlled by the (*S*)-configuration at the stereocenter on the heterocycle, that is (5*S*,α*R*)-**5a** and (5*S*,α*S*)-**5b** versus (5*R*,α*R*)-**5b** and (5*R*,α*S*)-**5a**, rather than by the configuration of the stereocenter bearing the secondary alcohol moiety, which usually plays a crucial role in the recognition process by the β-AR subtypes. As a matter of fact, the absolute configuration of the eutomer is (*R*)- for the β-stimulating arylethanolamines and, due to a change in the priority rules, is (*S*)- for the aryloxypropanolamines provided with β-blocking properties.

The isoxazolinyl aminoalcohols (+)-**5a**, (−)-**5a**, (+)-**5b**, (+)-**6a**, and (+)-**7a** showed reasonable binding affinity for at least one β-AR subtype and were, therefore, submitted to a functional assay along with reference compounds (±)-**1**-(±)-**4**. Stimulation of adenylyl cyclase (AC) was tested in membranes from stably transfected CHO cells with similar receptor expression for each β-AR subtype.[14] All compounds were tested for activity at a concentration of 100 μM. The maximal stimulation obtained with isoproterenol (100%) and the basal activity (0%) were used as reference points to evaluate the efficacy of the test compounds. From our previous characterization of the transfected CHO cell clones used in this study we know that EC_{50} values for agonists tend to be lower than the corresponding K_i values from binding experiments.[14] Therefore, if ligands were used at K_i concentrations a more than 50% signal is expected from a compound with agonistic activity. The values for isoxazolinyl derivatives in Table 2 showing low or no efficacy indicate, therefore, that the compounds with detectable binding affinity are indeed antagonists at all three β-AR subtypes. The data in Table 2 confirm that Broxaterol (±)-**2** is a partial agonist at β_2- and β_3- receptors, whereas it is an antagonist at β_1-receptors.[13,14]

Table 2. Adenylyl cyclase responses of human β-adrenergic receptor subtypes to selected ligands

Compound	Efficacy % (isoproterenol = 100%)					
	β_1-rec.	SEM	β_2-rec.	SEM	β_3-rec.	SEM
(+)-**5a**	3.9	4.0	1.2	4.1	1.1	5.4
(−)-**5a**	6.0	7.3	12.0	2.6	—[a]	—[a]
(+)-**5b**	0.8	3.5	5.7	4.9	—[a]	—[a]
(+)-**6a**	1.4	4.6	−11.9	5.3	—[a]	—[a]
(+)-**7a**	4.6	1.8	13.7	4.0	—[a]	—[a]
(±)-**1**	2.9	6.2	−35.0	6.7	−1.0	5.1
(±)-**2**	8.4	2.5	87.5	6.7	41.8	3.2
(±)-**3**	−2.5	7.2	36.1	7.6	20.3	2.3
(±)-**4**	−13.0	9.7	47.0	7.0	14.5	0.5

Membranes were prepared from cells with comparable receptor expression level. Adenylyl cyclase stimulation represents the percentage of maximal stimulation achieved by 100 μM isoproterenol (positive values) or percent inhibition of basal activity (negative values).

[a] Efficacy cannot be determined for compounds showing no binding affinity within detection limit.

The same holds true for the isoxazoles (±)-**3** and (±)-**4**, although the partial agonistic activity at β_3-receptors is marginal. As a consequence, the functional β_1-selectivity which characterizes the 3-bromo isoxazole (±)-**2** is a property shared, to a lesser extent, by the 3-isopropenyl [(±)-**3**] and the 3-cyclopropyl [(±)-**4**] analogs demonstrating that the presence of the heteroaromatic moiety is a prerequisite for agonistic activity at β_2- and, to some degree, at β_3-ARs. Such a property is not shared by the corresponding isoxazoline analogs. Interestingly, Falintolol (±)-**1**, which is structurally related to the Δ^2-isoxazoline derivatives **5-7**, shows inverse agonism at β_2-AR subtypes.

4. Conclusions

The present results put in evidence that, in a group of structurally related ligands for the β-ARs, the replacement of the isoxazole ring with the Δ^2-isoxazoline moiety produced some reduction of the binding affinity at β-adrenergic receptors and, at the same time, turned the mixed β_1-antagonists and β_2/β_3-agonists into neutral antagonists at all three β-ARs. The novel enantiopure isoxazolinyl aminoalcohols showed dramatically different binding characteristics in particular at β_1- and β_2-receptors, depending on the stereochemistry of the compounds. Binding affinity was controlled by the (*S*)-configuration at the stereocenter located on the heterocycle rather than by the configuration of the stereocenter bearing the secondary alcohol group. This observation makes these compounds interesting tools for studies devoted to ligand recognition and activation of β-ARs.

5. Experimental

5.1. Materials and methods

Cyclopropancarbaldehyde oxime,[12] BTMA ICl_4,[19] (*S*)-(+)-**10**[16,17] and (*R*)-(−)-**10**,[16,18] 2-chloro-2-(hydroxyimino)-acetate **14**,[25] (±)-Falintolol oxalate (±)-**1**,[6] (±)-Broxaterol **2** (free base),[11] isoxazoles (±)-**3** and (±)-**4** (free bases),[12] and the 1:1 oxalates of Δ^2-isoxazolines (+)-**5a**/(−)-**5a** and (−)-**5b**/(+)-**5b**[15] were all prepared according to published procedures. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (^{1}H, 300.063; ^{13}C, 75.451 MHz) in $CDCl_3$ solutions; chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz. The NMR data of final compounds refer to the corresponding free bases. Melting points were determined on a Mod. B 540 Büchi apparatus and are uncorrected. Liquid compounds were characterized by the oven temperature for bulb to bulb distillations. Rotary power determinations were carried out with a Perkin-Elmer 241 polarimeter coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 F254 aluminum sheets: spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Microanalyses (C, H, and N) of new compounds agreed with the theoretical value ±0.4%.

The radioligand (−)-3-[^{125}I]Iodocyanopindolol ([^{125}I]CYP) was purchased from Amersham Biosciences (specific activity, 2200 Ci/mmol). [α-^{32}P]ATP was from Perkin-Elmer LifeScience. Cell culture media and fetal calf serum were from PanSystems, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine, and G-418 were purchased from Gibco-Life Technologies. All other materials were from sources as described earlier.[14]

5.2. Chemical experimental section

5.2.1. 2-*tert*-Butylamino-1-(3-cyclopropyl-4,5-dihydroisoxazol-5-yl)-ethanols (+)-6a/(−)-6a and (+)-6b/(−)-6b. (A) To a solution of cyclopropancarbaldehyde oxime **8**[12] (4.50 g, 52.88 mmol) in dichloromethane (100 mL) was added BTMA ICl_4[19] (22.15 g, 52.88 mmol). The suspension disappeared in about 15 min upon vigorous stirring at rt. The resulting yellow solution was further stirred at rt for an additional 45 min and then diethyl ether (400 mL) was added. The precipitate was filtered off and evaporation of the filtrate afforded 5.30 g (84% yield) of crude cyclopropancarbohydroximoyl chloride **9** as a yellowish oil, which was used without further purification.

(B) To an ethyl acetate solution (250 mL) of crude hydroximoyl chloride **9** (5.30 g, 44.33 mmol) and (*S*)-(+)-**10**[16] (6.0 g, 46.81 mmol) was added solid sodium bicarbonate (18.50 g, 0.22 mol). After stirring at rt for about 3 d, the slurry was poured into water, the organic layer separated and the aqueous phase was extracted with ethyl acetate (3× 50 mL). The pooled organic extracts were dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. A silica gel column chromatography of the residue (eluant: 10% ethyl acetate/petroleum ether) gave 3.32 g of the *anti* isomer (+)-**11a** and 0.900 g of the *syn* isomer (−)-**11b** (45% overall yield).

Compound (+)-**11a** (5*S*,α*R*): colorless prisms (from petroleum ether/ethyl acetate), mp 70.5–72.5 °C. R_f 0.40 (eluant: 30% ethyl acetate/cyclohexane); $[\alpha]_D^{20}$ +91.8 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 0.76 (m, 2H), 0.90 (m, 2H), 1.34 (s, 3H), 1.41 (s, 3H), 1.80 (m, 1H), 2.76 (dd, 1H, *J* = 6.3 and 16.9), 2.88 (dd, 1H, *J* = 9.9 and 16.9), 3.90 (dd, 1H, *J* = 4.4 and 8.0), 3.98 (m, 1H), 4.09 (dd, 1H, *J* = 6.1 and 8.0), 4.42 (ddd, 1H, *J* = 6.3, 6.6 and 9.9). Anal. Calcd for $C_{11}H_{17}NO_3$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.22; H, 8.38; N, 6.29.

Compound (−)-**11b** (5*R*,α*R*): colorless prisms (from petroleum ether/ethyl acetate), mp 73–74.5 °C. R_f 0.29 (eluant: 30% ethyl acetate/cyclohexane); $[\alpha]_D^{20}$ −120.9 (*c* 0.98, $CHCl_3$); ^{1}H NMR: 0.77 (m, 2H), 0.89 (m, 2H), 1.35 (s, 3H), 1.43 (s, 3H), 1.77 (m, 1H), 2.67 (dd, 1H, *J* = 8.0 and 16.9), 2.81 (dd, 1H, *J* = 10.6 and 16.9), 3.79 (dd, 1H, *J* = 6.7 and 8.0), 4.04 (dd, 1H, *J* = 8.0 and 8.0), 4.24 (m, 1H), 4.60 (ddd, 1H, *J* = 4.8, 8.0 and 10.6). Anal. Calcd for $C_{11}H_{17}NO_3$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.30; H, 7.98; N, 6.41.

Cycloaddition to (*R*)-(−)-**10** along with the above-described protocol allowed isolation of stereoisomers (−)-**11a** and (+)-**11b** in comparable yields.

Compound (−)-**11a** (5*R*,α*S*): mp 70–72 °C; $[\alpha]_D^{20}$ −90.3 (*c* 0.99, $CHCl_3$). Anal. Calcd for $C_{11}H_{17}NO_3$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.25; H, 7.90; N, 6.55.

Compound (+)-**11b** (5*S*,α*S*): mp 73–74.5 °C; $[\alpha]_D^{20}$ +118.9 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{11}H_{17}NO_3$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.65; H, 8.07; N, 6.38.

(C) A mixture of (+)-**11a** (1.40 g, 6.63 mmol) and 1.0 g of Amberlite IR-120 (plus) in 150 mL methanol was stirred and heated at reflux for 2 h. The reaction mixture was filtered and concentrated at reduced pressure. Column chromatography of the residue on silica gel afforded 1.03 g (91% yield) of the desired diol (+)-**12a**.

Compound (+)-**12a** (5*S*,α*R*): colorless leaflets (from diisopropyl ether), mp 84–85 °C. R_f 0.30 (eluant: ethyl acetate); $[\alpha]_D^{20}$ +113.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 0.77 (m, 2H), 0.90 (m, 2H), 1.77 (m, 1H), 2.08 (br s, 1H), 2.58 (br s, 1H), 2.76 (dd, 1H, *J* = 10.3 and 17.0), 2.89 (dd, 1H, *J* = 8.4 and 17.0), 3.59 (dd, 1H, *J* = 5.9 and 11.4), 3.67–3.85 (m, 2H), 4.51 (ddd, 1H, *J* = 5.0, 8.4 and 10.3). Anal. Calcd for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.40; H, 7.70; N, 7.92.

The same procedure carried out on 3-cyclopropyl-Δ^2-isoxazolines (−)-**11a**, (−)-**11b**, and (+)-**11b** produced diols (−)-**12a**, (−)-**12b**, and (+)-**12b** in similar yields.

Compound (−)-**12a** (5*R*,α*S*): mp 84–85 °C; $[\alpha]_D^{20}$ −110.9 (*c* 0.99, $CHCl_3$). Anal. Calcd for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18. Found: C, 55.88; H, 7.47; N, 8.39.

Compound (−)-**12b** (5*R*,α*R*): colorless prisms (from diisopropyl ether), mp 92.5–93.5 °C. R_f 0.23 (eluant: ethyl acetate); $[\alpha]_D^{20}$ −166.7 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 0.78 (m, 2H), 0.91 (m, 2H), 1.78 (m, 1H), 2.50 (br s, 2H), 2.77 (dd, 1H, *J* = 8.0 and 16.7), 2.86 (dd, 1H, *J* = 10.0 and 16.7), 3.59 (m, 1H), 3.71 (m, 2H), 4.60 (ddd, 1H, *J* = 4.6, 8.0 and 10.0). Anal. Calcd for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.27; H, 7.90; N, 8.37.

Compound (+)-**12b** (5*S*,α*S*): mp 92–93 °C; $[\alpha]_D^{20}$ +165.2 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_8H_{13}NO_3$: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.25; H, 7.35; N, 8.03.

(D) To a solution of 0.500 g (2.92 mmol) of (+)-**12a** in dichloromethane (7.5 mL) were added toluene-4-sulfonic acid monohydrate (7.5 mg) and trimethyl orthoacetate (445 μL, 3.53 mmol). After stirring at rt for 0.5 h, the volatiles were evaporated at reduced pressure and the residue was dissolved in dichloromethane (7.5 mL). Trimethylchlorosilane (525 μL, 4.14 mmol) was then added and the reaction mixture was stirred for 4 h at rt. After removal of the solvent, the residue was taken up with MeOH (15 mL) and treated with potassium carbonate (0.810 g). The suspension was vigorously stirred for 1 h and then 30 mL of a saturated aqueous solution of ammonium chloride was added. The reaction mixture was extracted with dichloromethane (3× 15 mL) and, after the usual work-up, the residue was purified by column chromatography (eluant: 20% ethyl acetate/petroleum ether), affording 0.295 g (66% yield) of the desired epoxide (+)-**13a**.

Compound (+)-**13a** (5*S*,α*R*): colorless oil, bp 140–145 °C/1.5 mm Hg. R_f 0.67 (eluant: 30% ethyl acetate/cyclohexane); $[\alpha]_D^{20}$ +121.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 0.77 (m, 2H), 0.87 (m, 2H), 1.79 (m, 1H), 2.58 (m, 1H), 2.65 (dd, 1H, *J* = 6.9 and 16.7), 2.83 (m, 1H), 2.86 (dd, 1H, *J* = 10.2 and 16.7), 3.07 (m, 1H), 4.46 (ddd, 1H, *J* = 4.7, 6.9, and 10.2). Anal. Calcd for $C_8H_{11}NO_2$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.39; H, 6.91; N, 9.39.

Epoxides (−)-**13a**, (−)-**13b**, and (+)-**13b** were prepared from diols (−)-**12a**, (−)-**12b**, and (+)-**12b** in comparable yields through the same sequence of steps.

Compound (−)-**13a** (5*R*,α*S*): colorless oil, bp 140–145 °C/1.5 mm Hg. $[\alpha]_D^{20}$ −120.2 (*c* 1.01, $CHCl_3$). Anal. Calcd for $C_8H_{11}NO_2$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.59; H, 7.50; N, 8.92.

Compound (−)-**13b** (5*R*,α*R*): colorless oil, bp 140–145 °C/1.5 mm Hg. R_f 0.25 (eluant: 40% ethyl acetate/cyclohexane); $[\alpha]_D^{20}$ −132.5 (*c* 0.98, $CHCl_3$); ^{1}H NMR: 0.78 (m, 2H), 0.89 (m, 2H), 1.77 (m, 1H), 2.71 (dd, 1H, *J* = 7.5 and 16.7), 2.78 (m, 2H), 2.92 (dd, 1H, *J* = 10.5 and 16.7), 3.11 (m, 1H), 4.55 (ddd, 1H, *J* = 4.6, 7.5 and 10.5). Anal. Calcd for $C_8H_{11}NO_2$: C, 62.73; H, 7.24; N, 9.14. Found: C, 63.0; H, 7.15; N, 8.88.

Compound (+)-**13b** (5*S*,α*S*): colorless oil, bp 140–145 °C/1.5 mm Hg. $[\alpha]_D^{20}$ +135.0 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_8H_{11}NO_2$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.60; H, 7.41; N, 9.35.

(E) A stirred solution of (+)-**13a** (250 mg, 1.63 mmol) and *tert*-butylamine (1 mL, 9.5 mmol) in methanol (10 mL) was refluxed until TLC evidenced the disappearance of the starting material (about 2 h). The solvent and excess reagent were removed under vacuum, then the residue was dissolved in 3 N HCl (15 mL) and washed with diethyl ether (3× 10 mL). The aqueous layer was alkalinized with solid sodium carbonate and extracted with dichloromethane (3× 10 mL). After the usual work-up, the colorless oily residue of the pooled organic extracts (314 mg, 85% yield) was dissolved in ethanol and treated with a threefold excess of anhydrous oxalic. The corresponding oxalate, which precipitated immediately, was recovered by suction filtration.

Compound (+)-**6a** (5*S*,α*R*): thick colorless oil. R_f 0.23 (eluant: 40% methanol/chloroform); ^{1}H NMR: 0.71 (m, 2H), 0.82 (m, 2H), 1.03 (s, 9H), 1.71 (m, 1H), 2.18 (br s, 2H), 2.46 (dd, 1H, *J* = 7.7 and 12.0), 2.73 (dd, 1H, *J* = 3.8 and 9.1), 2.75–2.84 (m, 2H), 3.45 (m, 1H), 4.30 (ddd, 1H, *J* = 6.7, 7.9 and 9.7); ^{13}C NMR: 6.9 (CH_2, cyclopropyl), 9.9 (*C*H, cyclopropyl), 30.0 (C*Me*$_3$), 38.1 (C-4), 45.4 (CH_2N), 51.3 (NH*C*), 71.4

(*C*HOH), 82.3 (C-5), 162.4 (C-3). (+)-**6a** (5*S*,α*R*) × $C_2H_2O_4$: colorless prisms (from 2-propanol), mp 158.5–160 °C; $[\alpha]_D^{20}$ +112.3 (*c* 1.0, MeOH). Anal. Calcd for $C_{14}H_{24}N_2O_6$: C, 53.15; H, 7.65; N, 8.86. Found: C, 53.39; H, 7.54; N, 8.97.

Isomeric aminoalcohols (−)-**6a**, (−)-**6b**, and (+)-**6b** were obtained in comparable yields from epoxides (−)-**13a**, (−)-**13b**, and (+)-**13b**, respectively.

Compound (−)-**6a** (5*R*,α*S*) × $C_2H_2O_4$: colorless prisms (from 2-propanol), mp 158.5–160 °C; $[\alpha]_D^{20}$ −111.6 (*c* 1.0, MeOH). Anal. Calcd for $C_{14}H_{24}N_2O_6$: C, 53.15; H, 7.65; N, 8.86. Found: C, 52.95; H, 7.38; N, 8.69.

Compound (−)-**6b** (5*R*,α*R*): thick colorless oil. R_f 0.15 (eluant: 40% methanol/chloroform); ^{1}H NMR: 0.76 (m, 2H), 0.87 (m, 2H), 1.08 (s, 9H), 1.76 (m, 1H), 2.16 (br s, 2H), 2.69 (m, 2H), 2.81 (m, 2H), 3.53 (m, 1H), 4.50 (ddd, 1H, *J* = 3.5, 8.3 and 8.3); ^{13}C NMR: 6.9 (*C*H$_2$, cyclopropyl), 9.9 (*C*H, cyclopropyl), 29.9 (C*Me*$_3$), 38.0 (C-4), 45.6 (*C*H$_2$N), 51.3 (NH*C*), 71.8 (*C*HOH), 82.5 (C-5), 162.4 (C-3). (−)-**6b** (5*R*,α*R*) × $C_2H_2O_4$: colorless prisms (from 2-propanol/diethyl ether), mp 122–123.5 °C; $[\alpha]_D^{20}$ −100.1 (*c* 1.0, MeOH). Anal. Calcd for $C_{14}H_{24}N_2O_6$: C, 53.15; H, 7.65; N, 8.86. Found: C, 52.89; H, 7.44; N, 9.08.

Compound (+)-**6b** (5*S*,α*S*) × $C_2H_2O_4$: colorless prisms (from 2-propanol/diethyl ether), mp 122–123.5 °C; $[\alpha]_D^{20}$ +102.9 (*c* 1.0, MeOH). Anal. Calcd for $C_{14}H_{24}N_2O_6$: C, 53.15; H, 7.65; N, 8.86. Found: C, 53.21; H, 7.82; N, 8.75.

5.2.2. 2-*tert*-Butylamino-1-(3-vinyl-4,5-dihydroisoxazol-5-yl)-ethanols (+)-7a and (−)-7b. (A) To an ethyl acetate solution (400 mL) of ethyl 2-chloro-2-(hydroxyimino)-acetate **14**[25] (7.5 g, 49.49 mmol) and (*S*)-(+)-**10**[16] (6.0 g, 46.81 mmol) was added solid sodium bicarbonate (20.17 g, 0.24 mol). After stirring at rt for about 1 d, the slurry was poured into water and treated as described for the previous cycloaddition. A silica gel column chromatography of the residue (eluant: 10% ethyl acetate/petroleum ether) gave 4.94 g of the *anti* isomer (+)-**15a** and 1.92 g of the *syn* isomer (−)-**15b** (57% overall yield).

Compound (+)-**15a** (5*S*,α*R*): $[\alpha]_D^{20}$ +93.8 (*c* 1.0, $CHCl_3$) {lit.[20] $[\alpha]_D^{24}$ +95.1 (*c* 1.88, $CHCl_3$)}. Analytical and spectroscopic data matched those known from the literature.[20]

Compound (−)-**15b** (5*R*,α*R*): $[\alpha]_D^{20}$ −99.7 (*c* 0.98, $CHCl_3$) {lit.[20] $[\alpha]_D^{24}$ −93.6 (*c* 0.69, $CHCl_3$)}. Analytical and spectroscopic data matched those known from the literature.[20]

(B) To an ice-cooled solution of (+)-**15a** (3.0 g, 12.33 mmol) in absolute EtOH (150 mL) was slowly added sodium borohydride (875 mg, 23.13 mmol). After stirring at rt for about 5 h, the crude reaction mixture was concentrated at reduced pressure, filtered on a short Celite pad, and submitted to a silica gel column chromatography (eluant: 40% petroleum ether/ethyl acetate), affording 2.33 g (94% yield) of the primary alcohol (+)-**16a**.

Compound (+)-**16a** (5*S*,α*R*): colorless leaflets (from diisopropyl ether), mp 47–49 °C. R_f 0.53 (eluant: ethyl acetate); $[\alpha]_D^{20}$ +96.4 (*c* 0.98, $CHCl_3$); ^{1}H NMR: 1.32 (s, 3H), 1.41 (s, 3H), 2.58 (br s, 1H), 3.03 (dd, 1H, *J* = 7.5 and 17.6), 3.16 (dd, 1H, *J* = 10.2 and 17.6), 3.84 (m, 1H), 4.08 (m, 2H), 4.38 (s, 2H), 4.55 (ddd, 1H, *J* = 6.4, 7.5 and 10.2). Anal. Calcd for $C_9H_{15}NO_4$: C, 53.72; H, 7.51; N, 6.96. Found: C, 53.95; H, 7.25; N, 6.71.

The same procedure applied to ester (−)-**15b** gave alcohol (−)-**16b** with a comparable yield.

Compound (−)-**16b** (5*R*,α*R*): colorless leaflets (from diisopropyl ether), mp 43–45 °C. R_f 0.41 (eluant: ethyl acetate); $[\alpha]_D^{20}$ −120.8 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 1.33 (s, 3H), 1.41 (s, 3H), 2.30 (br s, 1H), 2.94 (dd, 1H, *J* = 8.0 and 17.7), 3.12 (dd, 1H, *J* = 10.7 and 17.7), 3.80 (dd, 1H, *J* = 7.1 and 8.6), 4.05 (dd, 1H, *J* = 7.1 and 7.1), 4.23 (m, 1H), 4.40 (s, 2H), 4.69 (ddd, 1H, *J* = 5.0, 8.0 and 10.7). Anal. Calcd for $C_9H_{15}NO_4$: C, 53.72; H, 7.51; N, 6.96. Found: C, 53.88; H, 7.30; N, 7.18.

(C) A suspension of (+)-**16a** (2.0 g, 9.94 mmol), pyridinium chlorochromate (10.71 g, 49.70 mmol), and sodium acetate (3.26 g, 39.76 mmol) in dichloromethane (120 mL) was stirred at rt until disappearance of the starting material (about 1 h). Celite was added followed by diethyl ether (100 mL), the resulting slurry was filtered under vacuum under a short silica pad and washed with diethyl ether. The filtrate was treated with a saturated solution of copper sulfate, and the organic phase was dried and evaporated. The crude intermediate aldehyde (+)-**17a** was submitted to the following step without further purification.

To an ice-cooled stirred suspension of potassium *tert*-butoxide (2.17 g, 19.31 mmol) in anhydrous toluene (200 mL) was added portionwise methyltriphenylphosphonium bromide (7.38 g, 20.66 mmol). After heating at reflux for 1 h, the suspension was cooled at rt. A solution of crude (+)-**17a** (1.65 g, 8.28 mmol) in toluene (10 mL) was then added dropwise. The mixture was stirred at rt for about 30 min until disappearance of the starting material; the progress of the reaction was monitored by TLC (eluant: 20% ethyl acetate/petroleum ether). Acetone (15 mL) and water (50 mL) were then added, the organic phase was separated and the aqueous phase was extracted with ether (3× 50 mL). After the usual work-up, the residue was submitted to column chromatography (eluant: 10% ethyl acetate/petroleum ether) affording 1.10 g (56% overall yield) of the desired *anti* 3-vinyl-Δ^2-isoxazoline (+)-**18a**.

Compound (+)-**18a** (5*S*,α*R*): colorless oil, bp 115–120 °C/1.5 mm Hg. R_f 0.38 (eluant: 20% ethyl acetate/petroleum ether); $[\alpha]_D^{20}$ +137.5 (*c* 1.10, $CHCl_3$); ^{1}H NMR: 1.32 (s, 3H), 1.40 (s, 3H), 3.08 (dd, 1H, *J* = 7.1 and 16.8), 3.17 (dd, 1H, *J* = 10.0 and 16.8), 3.91 (dd,

1H, J = 4.3 and 7.9), 4.02 (m, 1H), 4.09 (dd, 1H, J = 6.1 and 7.9), 4.54 (ddd, 1H, J = 6.1, 7.1 and 10.0), 5.48 (d, 1H, J = 17.9), 5.53 (d, 1H, J = 10.7), 6.64 (dd, 1H, J = 10.7 and 17.9). Anal. Calcd for $C_{10}H_{15}NO_3$: C, 60.90; H, 7.67; N, 7.10. Found: C, 60.57; H, 7.98; N, 6.95.

Alkene (−)-**18b** was obtained quite similarly by applying the above-described protocol to alcohol (−)-**16b**.

Compound (−)-**18b** (5*R*,α*R*): colorless oil, mp 110–115 °C/1.5 mm Hg. R_f 0.31 (eluant: 20% ethyl acetate/petroleum ether); $[\alpha]_D^{20}$ −212.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR: 1.30 (s, 3H), 1.39 (s, 3H), 2.92 (dd, 1H, J = 8.2 and 16.8), 3.08 (dd, 1H, J = 10.7 and 16.8), 3.77 (dd, 1H, J = 6.9 and 8.0), 4.01 (dd, 1H, J = 8.0 and 8.0), 4.20 (m, 1H), 4.64 (ddd, 1H, J = 4.8, 8.2 and 10.7), 5.42 (d, 1H, J = 17.9), 5.52 (d, 1H, J = 10.7), 6.60 (dd, 1H, J = 10.7 and 17.9). Anal. Calcd for $C_{10}H_{15}NO_3$: C, 60.90; H, 7.67; N, 7.10. Found: C, 61.15; H, 7.40; N, 6.88.

(D) Acetonide (+)-**18a** (850 mg, 4.31 mmol) was converted into diol (+)-**19a** (596 mg, 88% yield) through the procedure above described for (+)-**11a**. Isomeric diol (−)-**19b** was prepared in a similar yield from (−)-**18b**.

Compound (+)-**19a** (5*S*,α*R*): colorless leaflets (from diisopropyl ether), mp 61.5–63 °C. R_f 0.42 (eluant: ethyl acetate); $[\alpha]_D^{20}$ +181.0 (*c* 1.05, $CHCl_3$); ^{1}H NMR: 2.08 (br s, 2H), 3.08 (dd, 1H, J = 10.7 and 16.7), 3.21 (dd, 1H, J = 8.3 and 16.7), 3.68 (dd, 1H, J = 5.9 and 11.3), 3.73–3.90 (m, 2H), 4.64 (ddd, 1H, J = 5.4, 8.3 and 10.7), 5.52 (d, 1H, J = 17.7), 5.60 (d, 1H, J = 10.8), 6.66 (dd, 1H, J = 10.8 and 17.7). Anal. Calcd for $C_7H_{11}NO_3$: C, 53.49; H, 7.05; N, 8.91. Found: C, 53.61; H, 7.22; N, 8.84.

Compound (−)-**19b** (5*R*,α*R*): colorless leaflets (from diisopropyl ether), mp 91.5–93.5 °C. R_f 0.30 (eluant: ethyl acetate); $[\alpha]_D^{20}$ −212.2 (*c* 1, $CHCl_3$); ^{1}H NMR: 1.60 (br s, 2H), 3.08 (dd, 1H, J = 8.6 and 16.9), 3.19 (dd, 1H, J = 12.1 and 16.9), 3.67 (m, 1H), 3.78 (m, 2H), 4.75 (ddd, 1H, J = 4.1, 8.6 and 12.1), 5.52 (d, 1H, J = 17.7), 5.60 (d, 1H, J = 10.8), 6.67 (dd, 1H, J = 10.8 and 17.7). Anal. Calcd for $C_7H_{11}NO_3$: C, 53.49; H, 7.05; N, 8.91. Found: C, 53.27; H, 6.85; N, 9.12.

(E) The sequence previously illustrated for the synthesis of (+)-**13a** and (−)-**13b** was applied to diols (+)-**19a** and (−)-**19b**, affording isomeric epoxides (+)-**20a** and (−)-**20b** in 54% and 72% yield, respectively.

Compound (+)-**20a** (5*S*,α*R*): colorless needles (from cyclohexane/ethyl acetate), mp 97.5–100 °C. R_f 0.42 (eluant: 30% ethyl acetate/petroleum ether); $[\alpha]_D^{20}$ +198.0 (*c* 1.10, $CHCl_3$); ^{1}H NMR: 2.63 (dd, 1H, J = 2.8 and 4.7), 2.84 (dd, 1H, J = 4.7 and 4.7), 2.96 (dd, 1H, J = 7.8 and 17.2), 3.12 (m, 1H), 3.14 (dd, 1H, J = 10.9 and 17.2), 4.60 (ddd, 1H, J = 4.8, 7.8 and 10.9), 5.47 (d, 1H, J = 17.8), 5.58 (d, 1H, J = 10.8), 6.66 (dd, 1H, J = 10.8 and 17.8). Anal. Calcd for $C_7H_9NO_2$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.38; H, 6.27; N, 9.92.

Compound (−)-**20b** (5*R*,α*R*): colorless prisms (from diisopropyl ether), mp 78–80 °C. R_f 0.31 (eluant: 30% ethyl acetate/petroleum ether); $[\alpha]_D^{20}$ −235.0 (*c* 1, $CHCl_3$); ^{1}H NMR: 2.80 (m, 2H), 3.04 (dd, 1H, J = 7.5 and 16.5), 3.15 (m, 1H), 3.22 (dd, 1H, J = 11.3 and 16.5), 4.64 (ddd, 1H, J = 4.8, 7.5 and 11.3), 5.49 (d, 1H, J = 17.9), 5.57 (d, 1H, J = 10.8), 6.66 (dd, 1H, J = 10.8 and 17.9). Anal. Calcd for $C_7H_9NO_2$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.63; H, 6.38; N, 9.98.

(F) Epoxides (+)-**20a** and (−)-**20b** were reacted with *tert*-butylamine as described above yielding amino alcohols (+)-**7a** and (−)-**7b** in 92% and 87% yield, respectively.

Compound (+)-**7a** (5*S*,α*R*): thick colorless oil. R_f 0.35 (eluant: 40% methanol/chloroform); ^{1}H NMR: 1.02 (s, 9H), 2.25 (br s, 2H), 2.49 (dd, 1H, J = 7.9 and 12.0), 2.76 (dd, 1H, J = 3.8 and 12.0), 3.03 (dd, 1H, J = 10.6 and 16.7), 3.10 (dd, 1H, J = 7.9 and 16.7), 3.50 (m, 1H), 4.44 (ddd, 1H, J = 6.7, 7.9 and 10.6), 5.44 (d, 1H, J = 17.6), 5.50 (d, 1H, J = 10.8), 6.60 (dd, 1H, J = 10.8 and 17.6); ^{13}C NMR: 29.5 (C*Me*$_3$), 35.5 (C-4), 44.7 (CH_2N), 50.7 (NH*C*), 70.8 (*C*HOH), 83.0 (C-5), 122.5 (CH=*C*H$_2$), 127.0 (*C*H=CH$_2$), 158.1 (C-3). (+)-**7a** (5*S*,α*R*) × $C_2H_2O_4$: colorless prisms (from 2-propanol), mp 157.5–159 °C; $[\alpha]_D^{20}$ +176.9 (*c* 1.0, MeOH). Anal. Calcd for $C_{13}H_{22}N_2O_6$: C, 51.65; H, 7.33; N, 9.27. Found: C, 51.59; H, 7.40; N, 9.22.

Compound (−)-**7b** (5*R*,α*R*): thick colorless oil (crystalline on standing). R_f 0.22 (eluant: 40% methanol/chloroform); ^{1}H NMR: 1.07 (s, 9H), 2.20 (br s, 2H), 2.71 (dd, 1H, J = 6.7 and 12.0), 2.77 (dd, 1H, J = 5.0 and 12.0), 3.12 (m, 2H), 3.60 (m, 1H), 4.64 (ddd, 1H, J = 3.5, 8.4 and 8.4), 5.49 (d, 1H, J = 17.6), 5.56 (d, 1H, J = 10.8), 6.66 (dd, 1H, J = 10.8 and 17.6); ^{13}C NMR: 29.4 (C*Me*$_3$), 35.4 (C-4), 44.8 (CH_2N), 50.5 (NH*C*), 71.2 (*C*HOH), 83.2 (C-5), 122.4 (CH=*C*H$_2$), 126.9 (*C*H=CH$_2$), 158.2 (C-3). (−)-**7b** (5*R*,α*R*) × $C_2H_2O_4$: colorless needles (from 2-propanol), mp 212–215.5 °C; $[\alpha]_D^{20}$ −242.7 (*c* 1.0, MeOH). Anal. Calcd for $C_{13}H_{22}N_2O_6$: C, 51.65; H, 7.33; N, 9.27. Found: C, 51.80; H, 7.25; N, 9.09.

5.3. Pharmacological experimental section

5.3.1. Cell culture and membrane preparation. CHO cells expressing the stably transfected human β-adrenergic receptor subtypes were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12), containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), and Geneticin (G-418, 0.2 mg/mL) at 37 °C in 5% CO_2/95% air. Details are described by Hoffmann et al.[14] Membranes for radioligand binding were prepared from frozen cells in a two-step procedure as described previously.[26] For the measurement of adenylyl cyclase a one-step procedure from fresh cells was used.[14,26]

5.3.2. Radioligand binding studies and adenylyl cyclase activity. Radioligand binding experiments were carried out as described recently.[14] In brief, membranes from CHO cells stably transfected with human β-adrenergic

receptor subtypes (β_1, β_2 about 5 μg, β_3 about 25 μg of protein) were incubated with the antagonist [^{125}I]CYP in a concentration of about 50 pM in the case of β_1- and β_2-receptors or about 80 pM [^{125}I]CYP for β_3-receptors. Assays were carried out in 50 mM Tris/HCl, pH 7.4 (assay buffer), containing 100 μM GTP, in a total volume of 200 μL. GTP was added to achieve monophasic binding curves for agonists. Membranes were incubated for 90 min at 30 °C. Bound ligand was separated from free ligand by filtration through Whatman GF/C filters, which were then washed three times with ice-cold assay buffer. Non-specific binding was determined in the presence of 10 μM alprenolol. K_i values were calculated by nonlinear curve fitting with the program SCTFIT.[27]

The activity of adenylyl cyclase in cell membranes was determined as described recently.[14] Briefly, the conversion of [α-^{32}P]ATP to [^{32}P]cAMP was measured in an assay mixture containing membranes from CHO cells expressing human β-adrenergic receptor subtypes (about 50 μg of protein), 100 μM cAMP, 0.2% BSA, 10 μM GTP, 100 μM ATP, 1 mM $MgCl_2$, 100 μM isobutylmethylxantine, and an ATP-regenerating system consisting of 15 mM phosphocreatine and 300 U/mL of creatine kinase in 50 mM Tris/HCl, pH 7.4. The reaction was allowed to proceed for 20 min at 37 °C. The reaction was stopped by precipitation with ZnAc and Na_2CO_3. After centrifugation, [^{32}P]cAMP and remaining [α-^{32}P]ATP in the supernatant were separated by chromatography over alumina (neutral) columns and the amount of [^{32}P]cAMP was determined in a β-counter. The efficacy of compounds under investigation was tested at a concentration of 100 μM and compared to the stimulation by 100 μM isoproterenol (100%) over basal adenylyl cyclase activity (0%). Inverse agonistic activity is expressed as percent inhibition of basal activity.

Acknowledgments

This work was financially supported by the Università degli Studi di Milano (FIRST 2003 and 2004). The expert technical assistance of Sonja Kachler and Nico Falgner is gratefully acknowledged. C.D.M. and K.-N.K. are grateful to the German-Italian Vigoni program for support of the present research project.

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