Synthesis and Biological Evaluation of Novel, Peripherally Selective Chromanyl Imidazolethione-Based Inhibitors of Dopamine β-Hydroxylase

Alexandre Beliaev,[†] David A. Learmonth,[†] and Patricio Soares-da-Silva*[,‡]

Laboratories of Chemistry and Pharmacology, Department of Research & Development, BIAL, 4745-457 S. Mamede do Coronado, Portugal

Received October 18, 2005

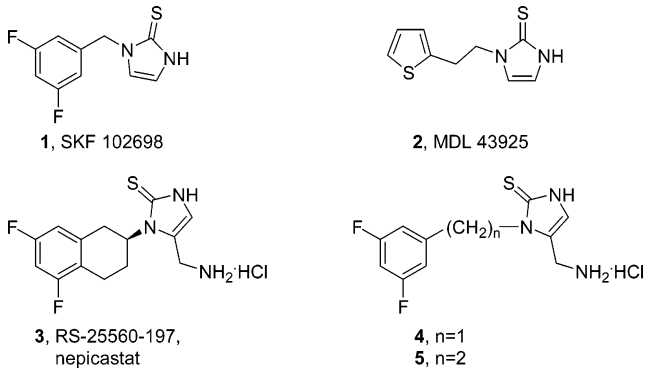
A novel series of dopamine β-hydroxylase (DBH) inhibitors was designed and synthesized incorporating modifications to the core structure of nepicastat **3**, with the principal aim of discovering potent DBH inhibitors exerting minimal effects on dopamine (DA) and noradrenaline (NA) levels in the central nervous system. This study resulted in the identification of a potent, peripherally selective DBH inhibitor, (*R*)-5-(2-aminoethyl)-1-(6,8-difluorochroman-3-yl)-1,3-dihydroimidazole-2-thione hydrochloride **54** (BIA 5-453). In experiments in mice and rats at T_{max} (9 h after administration), **54** reduced NA levels in a dose-dependent manner in both the left atrium and the left ventricle, with the maximal inhibitory effect attained at a dose of 100 mg/kg. In contrast to that found in the heart, **54** failed to affect NA tissue levels in the brain. Compound **54** is thus presented as a candidate for clinical evaluation for the treatment of chronic heart failure and hypertension.

Introduction

In recent years, interest in the development of inhibitors of dopamine β-hydroxylase (DBH; EC 1.14.17.1; dopamine β-monooxygenase) has centered on the hypothesis that inhibition of this enzyme may provide significant clinical improvements in patients suffering from cardiovascular disorders such as hypertension or chronic heart failure. The rationale for the use of DBH inhibitors is based on their capacity to inhibit the biosynthesis of noradrenaline (NA), which is achieved via enzymatic hydroxylation of dopamine (DA). Activation of neurohumoral systems, chiefly the sympathetic nervous system, is the principal clinical manifestation of congestive heart failure.[1] Congestive heart failure patients have elevated concentrations of plasma NA,[2] increased central sympathetic outflow,[3] and augmented cardiorenal NA spillover.[4] Prolonged and excessive exposure of myocardium to NA may lead to downregulation of cardiac β1-adrenoceptors, remodeling of the left ventricle, arrhythmias, and necrosis, all of which can diminish the functional integrity of the heart. Congestive heart failure patients who have high plasma concentrations of NA also have the most unfavorable long-term prognosis.[5] Of greater significance is the observation that plasma NA concentrations are already elevated in asymptomatic patients with no overt heart failure and can predict ensuing mortality and morbidity.[6] This implies that the activated sympathetic drive is not merely a clinical marker of congestive heart failure but may contribute to progressive worsening of the disease.

Inhibition of sympathetic nerve function with adrenoceptor antagonists appeared a promising approach, but unfortunately a significant proportion of patients do not tolerate the immediate hemodynamic deterioration that accompanies β-blocker treatment.[7] An alternative strategy for directly modulating sympathetic nerve function is to reduce the biosynthesis of NA via inhibition of DBH, the enzyme responsible for conversion of DA to NA in sympathetic nerves. This approach has several putative merits, such as gradual modulation, as opposed to abrupt inhibition of the sympathetic system, and causing increased release of DA, which can improve renal function such as renal vasodilation, diuresis, and natriuresis. Therefore it could be anticipated that inhibitors of DBH may provide significant clinical advantages over conventional β-blockers.

Chart 1

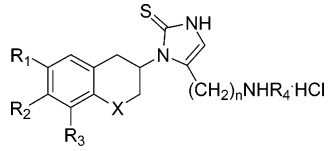


Several inhibitors of DBH have been thus far reported in the literature.[8−18] Early first- and second-generation examples such as disulfiram[8] and diethyl dithiocarbamate[9] or fusaric acid[11] and aromatic or alkyl thioureas[12] were found to be of low potency, exhibited poor selectivity for DBH, and caused side effects. However, the third generation of DBH inhibitors, such as 1,3-dihydroimidazole-2-thione derivatives SKF 102698 **1**[14] and MDL 43925 **2**,[15] were shown to have much greater potency (Chart 1). The most potent DBH inhibitor thus far reported in the literature is nepicastat (RS-25560-197, $IC_{50} = 9$ nM) **3**.[16−18] Although devoid of some of the problems associated with the first- and second-generation DBH inhibitors, nepicastat was found to cross the blood−brain barrier (BBB) and was thereby able to cause central as well as peripheral effects, a situation that could lead to undesired and potentially serious central nervous system (CNS) side effects of the drug. Therefore, there as yet remains an unfulfilled clinical requirement for a potent, nontoxic, and peripherally selective inhibitor of DBH that could be used for treatment of certain cardiovascular disorders. A DBH inhibitor with similar potency and selectivity to nepicastat but devoid of CNS effects (inability to cross the BBB) should

* Corresponding author: tel 351-22-9866100; fax 351-22-9866192; e-mail psoares.silva@bial.com.
[†] Laboratory of Chemistry.
[‡] Laboratory of Pharmacology.

10.1021/jm051051f CCC: $33.50
Published on Web 01/14/2006

Table 1. Effect of Synthesized Compounds on Dopamine β-Hydroxylase Activity in Vitro



target compd	starting amine **10**	intermediate (synth. route)	R_1	R_2	R_3	R_4	X	n	configuration	DBH activity, %[a]
3	**b**	N/A	F	H	F	H	CH_2	1	*S*	0.0 ± 0.3
39	**c**	**11** (A)	H	H	H	H	O	1	*R*	0.0 ± 1.9
40	**d**	**12** (A)	OH	H	H	H	O	1	*R*	17.8 ± 2.1
41	**e**	**13** (A)	F	H	F	H	O	1	*R*	4.1 ± 0.6
42	**a**	**19** (B)	H	H	H	H	CH_2	2	*S*	16.8 ± 4.8
43	**b**	**20** (B)	F	H	F	H	CH_2	2	*S*	1.6 ± 0.3
44	**c**	**21** (B)	H	H	H	H	O	2	*R*	3.0 ± 0.5
45	**f**	**22** (B)	F	H	H	H	O	2	*R*	8.0 ± 0.1
46	**g**	**23** (B)	OMe	H	H	H	O	2	*R*	54.5 ± 9.9
47	**d**	**24** (B)	OH	H	H	H	O	2	*R*	0.0 ± 0.6
48	**h**	**25** (B)	NO_2	H	H	H	O	2	*R*	94.8 ± 1.2
49	**i**	**26** (B)	H	H	F	H	O	2	*R*	6.9 ± 0.6
50	**j**	**27** (B)	H	H	OMe	H	O	2	*R*	6.9 ± 0.5
51	**k**	**28** (B)	H	H	OH	H	O	2	*R*	124.8 ± 6.5
52	**l**	**29** (B)	H	H	NO_2	H	O	2	*R*	53.2 ± 3.9
53	**m**	**30** (B)	F	F	H	H	O	2	*R*	9.4 ± 0.7
54	**e**	**31** (B)	F	H	F	H	O	2	*R*	3.3 ± 0.3
55	**n**	**32** (B)	F	H	F	H	O	2	*S*	50.2 ± 1.9
56	**o**	**33** (B)	Cl	H	OMe	H	O	2	*R*	36.7 ± 4.4
57	**p**	**34** (B)	OMe	H	Cl	H	O	2	*R*	94.0 ± 3.1
58	**q**	**35** (B)	OH	Bn	H	H	O	2	*R*	86.1 ± 2.7
59	**r**	**36** (B)	F	F	F	H	O	2	*R*	8.2 ± 0.7
60	**c**	**14** (A)	H	H	H	Me	O	2	*R*	2.2 ± 2.5
61	**d**	**15** (A)	OH	H	H	Me	O	2	*R*	15.5 ± 5.8
62	**e**	**16** (A)	F	H	F	Me	O	2	*R*	2.6 ± 1.6
63	**g**	**17** (A)	OMe	H	H	Bn	O	2	*R*	66.0 ± 4.5
64	**d**	**18** (A)	OH	H	H	Bn	O	2	*R*	4.5 ± 1.9
65	**s**	**37** (B)	OMe	H	H	H	S	2	*R,S*	99.4 ± 2.8
66	**t**	**38** (B)	OH	H	H	H	S	2	*R,S*	27.3 ± 0.4
73	**b**	**71** (N/A)	F	H	F	H	CH_2	3	*S*	8.1 ± 0.3

[a] Residual DBH activity calculated as a percent of NA concentration in a control experiment without inhibitor; mean ± SEM of three experiments per group.

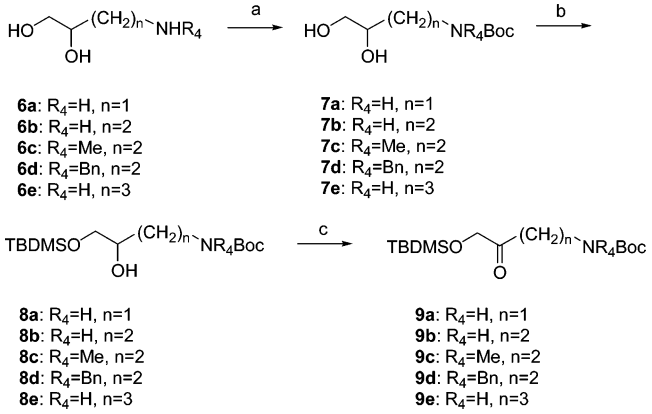
provide a significant improvement over the DBH inhibitors described so far.

The primary amino acid sequences for mouse,[19] rat,[20] bovine,[21−24] and human[25,26] DBH are known. However, the enzyme has not yet been crystallized and the 3D structure has not been determined, which has naturally created difficulties in the rational design of new inhibitors of DBH. A Hansch-type quantitative structure−activity relationship (QSAR) equation for the series of 1,3-dihydroimidazole-2-thione derivatives was obtained,[27] as well as a pharmacophore model for an enlarged group of the same type of compounds by means of the local minima method and CoMFA.[28] The above models, although having some predictive power, were developed for series of compounds unsubstituted at positions 4 and 5 of the imidazolethione moiety and thus cannot be applied to nepicastat **3** and related structures. The synthesis of the benzocycloalkyl imidazolethione-based DBH inhibitors including nepicastat have been described only in a patent,[16] and accordingly no SAR data are given. In a preliminary study, we confirmed that open-chain nepicastat analogues **4** and **5**, which contain essentially all of the key elements of the likely pharmacophore, were endowed with significantly reduced potency, which indicates that the unique spatial arrangement of the difluorobenzene, imidazolethione, and primary amino group in the molecule of **3** is important. Therefore, alternative structural modifications of lead compound **3** were considered, such as bioisosteric replacement of the methylene group of the cyclohexane moiety with oxygen or sulfur atoms and elongation of the linkage between the imidazole ring and the amino group as part of a search for peripherally selective DBH inhibitors. The effects on DBH inhibition caused by variation of substituents in the aromatic ring and at the amino group of the imidazolethione side chain was also investigated in combination with these changes.

Chemistry

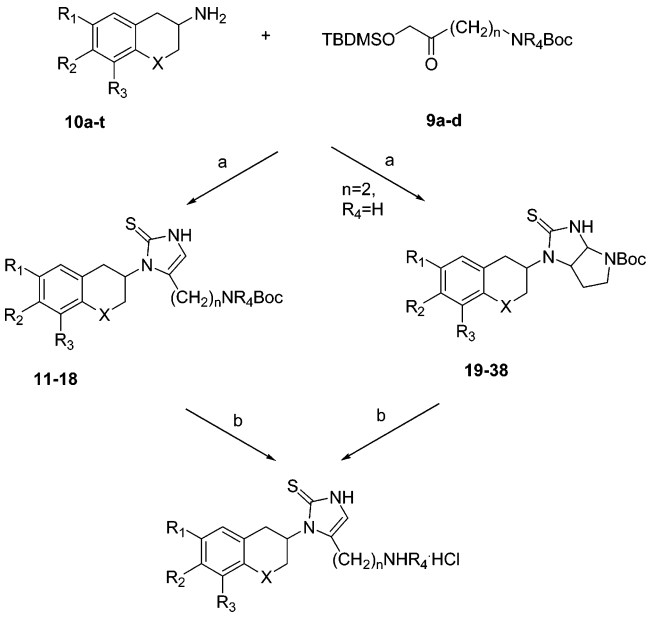
Various methods of the imidazole-2-thione ring synthesis are known.[29] However, many of them are not compatible with the substitution pattern of **3**. To prepare the compounds of Table 1, a novel method was developed using N-protected 1-((*O*-(*tert*-butyldimethylsilyl)oxy)methyl)-3-aminoalkyl ketones (**9a**−**d**, **70**). N-Boc-protected compounds **9a**−**e** were prepared starting from the corresponding amino diols (**6a**−**e**) by a modified procedure for the synthesis of **9a**[30] (Scheme 1). Cyclocondensation of the ketone **9a**, **9c**, or **9d** with amines **10c**−**e** or **10g** and potassium thiocyanate in the presence of acetic acid in EtOAc (Scheme 2) gave N-Boc-protected 5-aminoalkyl imidazole-2-thiones **11**−**18**, which upon cleavage with HCl/EtOAc afforded the target compounds **39**−**41** and **60**−**64**. In the case of N-Boc amino ethyl derivative **9b** and amines **10a**−**t**, hexahydropyrrolo[2,3-*d*]imidazole-2-thiones **19**−**38** were isolated in 40−70% yield. Apart from the asymmetric carbon atom in the chroman/tetrahydronaphthalene moiety, compounds **19**−**38** have two new chiral centers in the pyrroloimidazole fragment. The diastereoisomers of **26** could be separated by chromatography, while all others were obtained as mixtures of two isomers (four in the case of racemic thiochroman derivatives **37** and **38**). The structures indicated for **19**−**38** are based on 1D and 2D NMR spectroscopic data. Under the conditions employed

Scheme 1[a]



[a] Reagents and conditions: (a) Boc_2O, EtOH; (b) TBDMS-Cl, Et_3N, DMAP, DCM; (c) Dess−Martin periodinane, DCM.

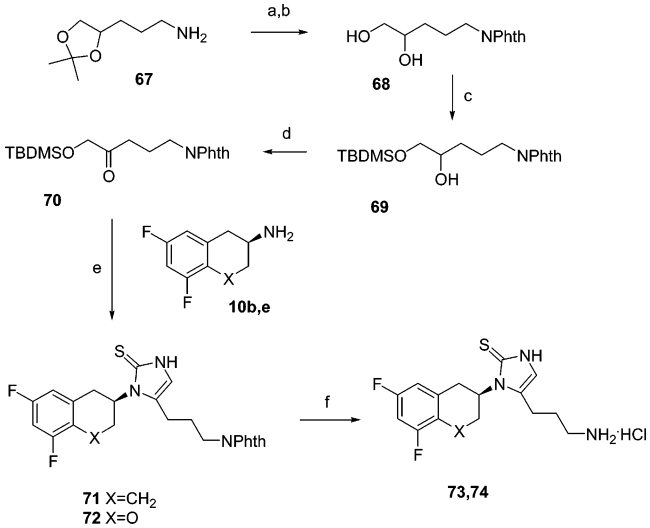
Scheme 2[a]



[a] Reagents and conditions: (a) KSCN, AcOH, EtOAc, reflux; (b) 1 M HCl, EtOAc.

for cleavage of the protecting group (1 N HCl/EtOAc), simultaneous opening of the pyrrolidine ring occurred to give the target compounds **42**−**59**, **65**, and **66** (Scheme 2). Chiral HPLC analysis of two enantiomers of the starting amines **10e** and **10n** and the final products **54** and **55** showed that the above synthetic sequence caused no detectable racemization. Cyclocondensation reactions of the amino propyl ketone **9e** resulted in very complex, inseparable mixtures, which did not allow isolation of any pure compound. For the synthesis of the aminopropyl derivatives **73** and **74**, the N-phthalyl-protected ketone **70** was prepared. Cyclocondensation followed by deprotection with sodium borohydride/acetic acid[31] afforded **73** and **74** (Scheme 3).

Scheme 3[a]



[a] Reagents and conditions: (a) *N*-carboethoxyphthalimide, Et_3N, EtOH; (b) 1 N HCl, THF; (c) TBDMS-Cl, Et_3N, DMAP, DCM; (d) Dess−Martin periodinane, DCM; (e) KSCN, AcOH, EtOAc, reflux; (f) $NaBH_4$/2-propanol/water, AcOH, reflux.

Results and Discussion

The inhibitory activity of the synthesized compounds and **3** against DBH was initially evaluated in vitro (Table 1). Incubation of SK-N-SH cells in the presence of increasing concentrations of DA resulted in a concentration-dependent formation of NA, yielding K_m (micromolar) and V_{max} (nanomoles per milligram of protein per hour) values of 20.6 ± 1.6 and 153.8 ± 4.4, respectively. From these kinetic parameters, a concentration of DA approaching saturation (50 mM) was chosen for use in inhibition studies. As can be seen from the results listed in Table 1, elongation of the linkage between imidazole ring and amino group of **3** as in **43** ($n = 2$) and **73** ($n = 3$) resulted in a consecutive loss of potency. Bioisosteric substitution of the methylene group of **3** with oxygen, as in **41**, resulted in a slightly greater decrease of activity than elongation of the side chain by one methylene group. Interestingly, the unsubstituted aromatic ring chromane derivative **39** is as potent as **3**, whereas the unsubstituted amino ethyl compound **42** was significantly less active than **43**. Introduction of a hydroxyl group in position 6 of the chroman system of **39** gave the less potent **40**.

The majority of compounds in Table 1 were designed by contemplating simultaneous bioisosteric substitution of the methylene group of **3** with oxygen (X = O) and elongation of the side chain by one methylene group ($n = 2$). The analogue **54** thus obtained, as the result of the above modifications, demonstrated slightly lower potency than the parent **3**. The unsubstituted derivative **44** showed activity similar to that of **54**. The best compound in this group was the 6-hydroxy chroman **47**, which is equipotent to **3**. Conversely, in the amino methyl series (X = O, $n = 1$) the 6-hydroxy derivative **40** was less potent than 6,8-difluoro and unsubstituted compounds. The data for compounds **46**, **48**, **57**, and **58** indicate that bulky substituents R_1 and R_2 in positions 6 and 7 of the chroman ring are not tolerated. The relatively high potency of the 8-OMe derivative **50** shows that position 8 has less steric restraints, although the presence instead of a free OH group at this position completely abolished inhibitory activity as seen for compound **51**. It is thus assumed that a hydrogen-bond donor in this region is very unfavorable for binding to the enzyme. Substitution of the primary amino group in the imidazolethione side chain with methyl (compounds **60**−**62**) or benzyl (compounds **63** and **64**) residues appeared neither beneficial nor detrimental in terms of inhibition. On the other hand, the correct configuration at the chiral center of the carbon atom at position 3 of the chroman ring is very important for inhibitory activity. Thus, compound **55** with the (*S*)-configuration at C(3) possesses only approximately $^1/_{100}$ the potency of the corresponding (*R*)-isomer **54**. This observation in part probably accounts for the lower activity of thiochroman derivatives **65** and **66**, which were

Table 2. Effect of Synthesized Compounds on Noradrenaline Tissue Levels in Mice[a]

	% of control ± SEM			
compd	6 h	9 h	18 h	24 h
3	63.05 ± 4.01	18.36 ± 3.42	47.87 ± 3.48	48.37 ± 3.87
41			24.64 ± 3.60	
43			52.79 ± 6.30	
44	86.85 ± 13.31	51.17 ± 1.47	80.30 ± 5.90	
47	75.04 ± 5.93	80.98 ± 3.77	85.24 ± 6.81	
54	49.42 ± 6.14	27.59 ± 3.38	12.98 ± 1.80	53.51 ± 7.27

[a] Compounds were administered at 100 mg/kg po. NA tissue concentration in the heart was calculated as a percent of the concentration in a control experiment without inhibitor and is presented as the mean ± SEM of four animals per group.

actually prepared and tested as racemic mixtures due to the surprising lack of effective synthetic methods for preparation of the optically pure intermediate thiochroman amines **10** (X = S). Furthermore, attempts to resolve the racemate through the use of several chiral acids also failed. Another explanation for the lower activity of **65** and **66** may be the increase in the ring size due to the length of the C−S bond, which in turn can lead to a slightly different orientation of the imidazolethione group and potentially decrease the compounds' ability to inhibit DBH. To test this hypothesis, the synthesis of homochiral seven-membered ring analogues was contemplated, but unfortunately this proved to be unsuccessful due to synthetic difficulties encountered with the preparation of the corresponding intermediate 2,3,4,5-tetrahydrobenzo[*b*]oxepin-4-ylamines.

Subsequently, compounds **41**, **43**, **44**, **47**, and **54** were chosen for further in vivo studies. Initial inhibition experiments were performed in mice with **3** as a reference substance. Each inhibitor (100 mg/kg) was delivered orally with water as vehicle, and NA tissue levels in the heart at 18 h were quantified by HPLC with electrochemical detection (Table 2). Compounds **41**, **43**, **54**, and **3** produced significant decreases in NA levels in heart. Compounds **44** and **47**, which were very potent in vitro, surprisingly demonstrated low activity in vivo at 18 h as well as at 6 and 9 h. A speculative reason for this could involve rapid metabolic deactivation of **44** and **47** by phase I oxidation of the aromatic ring followed by phase II conjugation, metabolic routes that would not be expected for the difluoroaromatic derivatives **41**, **43**, and **54**. As was shown in time-course experiments with **3** and the most active compound **54**, the time of maximum effect (T_{max}) for NA tissue reduction by active derivatives appears to be at 9 h postdose (Table 2). Thereafter, NA tissue levels recover, reaching 50% recovery of initial tissue levels at 24 h postdose.

As shown in mice, at T_{max} both **3** and **54** reduced NA levels in the left ventricle in a dose-dependent manner. For both **3** and **54**, the maximal inhibitory effect was attained at a dose of 100 mg/kg (Figure 1). In contrast to results in the heart, **54** failed to affect NA tissue levels in the parietal cortex of the brain, whereas **3** produced a dose-dependent decrease in NA levels in this area of the brain (Figure 2). This peripheral selectivity is likely to be associated with the reduced ability of **54** to cross the BBB. In part this can be explained by the lower lipophilicity of **54** caused by replacement of the more lipophilic benzylic methylene group with an oxygen atom. The presence of this oxygen atom could also be expected to influence other key molecular properties such as H-bonding capacity and polarity, which would be likely to contribute to the reduced ability of **54** to penetrate across the BBB.

In experiments in rats, the effects of both **3** and **54** upon NA were dependent on the dose administered and reached maxima

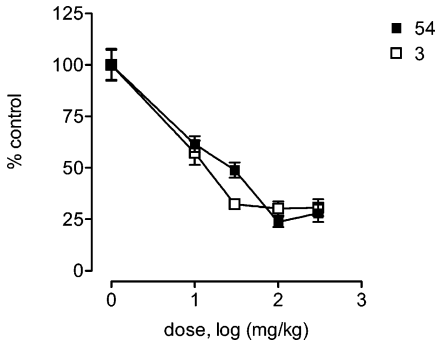


Figure 1. NA levels in the mouse left ventricle 9 h after oral administration of compounds **3** and **54**.

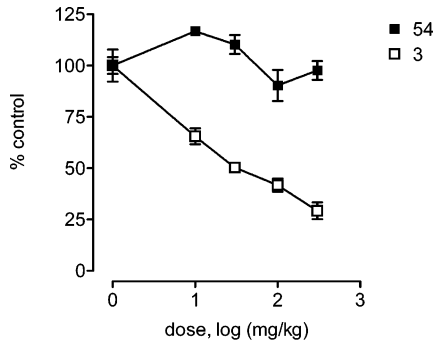


Figure 2. NA levels in the mouse brain parietal cortex 9 h after oral administration of compounds **3** and **54**.

Table 3. Effect of Compounds **3** and **54** on Noradrenaline Tissue Levels in Rats[a]

	% of control ± SEM			
compd	frontal cortex	parietal cortex	left atrium	left ventricle
3	43.90 ± 6.35	37.67 ± 7.05	79.74 ± 4.88	63.08 ± 16.43
54	107.46 ± 17.74	88.67 ± 5.54	62.54 ± 12.40	47.61 ± 12.34

[a] Compounds were administered at 100 mg/kg po. NA tissue concentration after 9 h was calculated as a percent of the concentration in a control experiment without inhibitor and is presented as the mean ± SEM of four animals per group.

at 9 h (data not shown). However, the inhibitory effects of **54** (100 mg/kg) upon NA levels in both the left atrium and the left ventricle were more pronounced than those elicited by **3** (100 mg/kg) (Table 3). Again, as observed in mice, **54** failed to affect NA tissue levels in the brain parietal cortex and the brain frontal cortex, whereas **3** produced a marked decrease in NA levels in these brain areas.

From the above data, it can be concluded that **54** (BIA 5-453), in stark contrast to **3**, exerts its inhibitory effects upon DBH almost exclusively in the periphery, being devoid of significant inhibitory effects in the brain. A combination of bioisosteric replacement of the methylene group with an oxygen atom and elongation of the side chain are beneficial for peripheral selectivity. Incorporation of these features has led to the discovery of **54**, a compound with high peripheral selectivity for DBH without loss of inhibitory activity.

Conclusion

Some of the synthesized compounds described herein are very potent DBH inhibitors and have potentially valuable pharmaceutical properties in the treatment of certain cardiovascular disorders such as chronic heart failure and hypertension, where a reduction in the enzymatic hydroxylation of DA to NA may be of therapeutic benefit. The possibility to use a long-acting DBH inhibitor with limited access to the brain, such as

compound **54**, opens promising new perspectives in the treatment of hypertension and chronic heart failure by improving potency and selectivity of DBH inhibition in the periphery.

Experimental Section

Chemistry. NMR spectra were recorded on a Bruker Avance DPX (400 MHz) spectrometer with solvent used as internal standard, and data are reported in the following order: chemical shift (ppm), number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), approximate coupling constant (*J*) in hertz, and assignment of a signal. Analytical TLC was performed on precoated silica gel plates (Merck 60 Kieselgel F 254), visualized with UV light and ninhydrin spray. Elemental analyses were performed on a Fisons EA 1110 CHNS instrument, and all analyses are consistent with theoretical values to within ±0.4%, unless otherwise indicated. Chiral HPLC was performed on Hewlett-Packard 1100 series instrument with diode array detection and a Chiralpak AD-H (250 × 4.6 mm) column. For compounds **10e,n**, MeOH/2-propanol (7:3) mixture with 0.2% *tert*-butylamine was used as a mobile phase (flow 0.5 mL/min); for compounds **54** and **55**, a MeOH/2-propanol (3:7) mixture with 0.4% *tert*-butylamine was used (flow 0.3 mL/ min). The target compounds melt around 200 °C with decomposition, and their melting points were not used for identification. Solvents and reagents were purchased from Aldrich, Merck, and Fluka and used as received unless otherwise noted.

Amino diol **6a** is available commercially, and **6b** and **6e** are known compounds.[31,32] Secondary amino diols **6c** and **6d** were prepared from **6b** by standard methods. Several of the amines **10a**−**t** are known compounds,[16,33−37] and others were prepared similarly according to the above methods.

(3,4-Dihydroxybutyl)carbamic acid *tert*-butyl ester 7b (General Procedure). To a stirred solution of **6b** (2.10 g, 20 mmol) in ethanol (50 mL) at room temperature was added di-*tert*-butyldicarbonate (4.80 g, 22 mmol) in one portion. The resulting mixture was stirred at room temperature for 2 h and then evaporated in vacuo and purified by column chromatography on silica, with ethyl acetate−petroleum ether mixture as eluent, to afford a colorless oil (3.12 g, 76%).

[4-((*tert*-Butyldimethylsilanyl)oxy)-3-hydroxybutyl]carbamic Acid *tert*-Butyl Ester 8b (General Procedure). To a stirred solution of **7b** (2.60 g, 12.7 mmol), triethylamine (2.03 mL, 14.50 mmol), and 4-(dimethylamino)pyridine (0.05 g, 0.4 mmol) in anhydrous dichloromethane (40 mL) at room temperature was added *tert*-butyldimethylchlorosilane (2.0 g, 13.17 mmol) in one portion. The resulting mixture was stirred at room temperature for 18 h, then washed with water and brine, and dried over anhydrous magnesium sulfate. Filtration and concentration in vacuo gave an oil, which was purified by column chromatography on silica, with ethyl acetate−petroleum ether mixture as eluent, to afford a colorless oil (3.53 g, 87%).

[4-((*tert*-Butyldimethylsilanyl)oxy)-3-oxobutyl]carbamic Acid *tert*-Butyl Ester 9b (General Procedure). To a solution of Dess−Martin periodinane (5.0 g, 11.8 mmol) in anhydrous dichloromethane (35 mL) at room temperature was added a solution **8b** (3.77 g, 11.8 mmol) in anhydrous dichloromethane. The resulting mixture was stirred at room temperature for 1 h, then evaporated in vacuo to a third of the initial volume, and applied to a column packed with silica. Elution with ethyl acetate−petroleum ether solvent mixture afforded a colorless oil (3.30 g, 88%).

(*R*)-[3-(6,8-Difluorochroman-3-yl)-2-thioxo-2,3-dihydro-1*H*-imidazol-4-ylmethyl]carbamic Acid *tert*-Butyl Ester 13 (General Procedure, Route A). A stirred mixture of (*R*)-6,8-difluorochroman-3-ylamine hydrochloride **10d** (0.22 g, 1.0 mmol), [3-((*tert*-butyldimethylsilanyl)oxy)-2-oxopropyl]carbamic acid *tert*-butyl ester **9a** (0.33 g, 1.1 mmol), potassium thiocyanate (0.11 g, 1.1 mmol), and acetic acid (0.3 mL, 5.0 mmol) in ethyl acetate (3 mL) was refluxed for 2 h, then cooled to room temperature and washed by sodium bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated in vacuo. The residue was purified by column chromatography over silica gel, with ethyl acetate−petroleum ether mixture as eluent, to afford a yellowish amorphous solid (0.23 g, 58%).

(*R*)-5-Aminomethyl-1-(6,8-difluorochroman-3-yl)-1,3-dihydroimidazole-2-thione Hydrochloride 41 (General Procedure, Route A). Compound **13** (0.21 g, 0.53 mmol) was dissolved in THF (3 mL), whereupon 12 N HCl was added (0.8 mL, 9.6 mmol) and the mixture was stirred for 2 h at room temperature. The solution was diluted with 2-propanol (20 mL), then evaporated to dryness in vacuo, and the residue was recrystallized from 2-propanol−ether to give beige crystals (0.102 g, 58%): δ_H (400 MHz, DMSO-d_6) 12.51 (1H, s, NH), 8.53 (3H, s, N^+H), 7.16 (1H, m, C7H), 7.11 (1H, d, C4′H), 6.93 (1H, m, C5H), 5.25 (1H, br s, C2H), 4.78 (1H, br s, C3H), 4.44 (2H, m, C2H and C4H), 4.11 (2H, br s, C1″H), 3.04 (1H, dd, C4H); δ_C (100 MHz, DMSO-d_6) 161.1 (CS), 154.9 (dd, J = 11.8 and 238.0, C6), 150.1 (dd, J = 13.3 and 248.0, C8), 138.0 (dd, J = 2.9 and 11.8, C8a), 124.6 (dd, J = 2.2 and 9.5, C5a), 124.1 (C5′), 116.5 (C4′), 110.9 (dd, J = 3.7 and 22.9, C5), 102.4 (dd, J = 22.1 and 27.3, C7), 64.2 (C2), 49.3 (C3), 32.0 (C1″), 26.8 (C4). Anal. ($C_{13}H_{14}ClF_2N_3OS\cdot0.5H_2O\cdot0.5C_3H_7OH$) C, H, N, S.

(*R*)-1-(6,8-Difluorochroman-3-yl)-2-thioxohexahydropyrrolo-[2,3-*d*]imidazole-4-carboxylic Acid *tert*-Butyl Ester 31 (General Procedure, Route B). A stirred mixture of (*R*)-6,8-difluorochroman-3-ylamine hydrochloride **10d** (1.68 g, 7.58 mmol), [4-((*tert*-butyldimethylsilanyl)oxy)-3-oxobutyl]carbamic acid *tert*-butyl ester **9b** (3.13 g, 9.85 mmol), potassium thiocyanate (0.96 g, 9.85 mmol), water (0.18 mL, 10 mmol), and acetic acid (3.0 mL, 50 mmol) in ethyl acetate (30 mL) was refluxed for 7 h, then cooled to room temperature and washed by sodium bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated in vacuo. The residue was purified by column chromatography on silica, with ethyl acetate−petroleum ether mixture as eluent, to afford a yellowish viscous oil (2.15 g, 69%).

(*R*)-5-(2-Aminoethyl)-1-(6,8-difluorochroman-3-yl)-1,3-dihydroimidazole-2-thione Hydrochloride 54 (General Procedure, Route B). Compound **31** (2.15 g) was dissolved in ethyl acetate (20 mL), 2 M HCl solution in ethyl acetate was added (20 mL, 40 mmol), and the mixture was stirred for 2 h at room temperature. The precipitate was collected by filtration and washed with ethyl acetate to give beige crystals (1.13 g, 65%): δ_H (400 MHz, DMSO-d_6) 12.28 (1H, s, NH), 8.12 (3H, s, N^+H), 7.15 (1H, m, C7H), 6.92 (2H, m, C5H and C4′H), 5.15 (1H, br s, C2H), 4.84 (1H, br s, C3H), 4.38 (2H, m, C2H and C4H), 2.94 (5H, br s, C4H and C1″H and C2″H); δ_C (100 MHz, DMSO-d_6) 160.7 (CS), 154.9 (dd, J = 11.6 and 238.2, C6), 150.2 (dd, J = 13.5 and 245.9, C8), 138.2 (dd, J = 2.6 and 11.6, C8a), 126.2 (C5′), 124.5 (dd, J = 1.3 and 9.7, C5a), 111.0 (dd, J = 3.2 and 23.2, C5), 113.4 (C4′), 102.6 (dd, J = 21.9 and 27.0, C7), 64.7 (C2), 49.0 (C3), 37.5 (C1″), 27.8 (C4), 22.5 (C2″). Anal. ($C_{14}H_{16}ClF_2N_3OS\cdot0.66CH_3CO_2C_2H_5$) C, H, N, S.

2-(4,5-Dihydroxypentyl)isoindole-1,3-dione 68. To a stirred solution of 3-(2,2-dimethyl-[1,3]dioxolan-4-yl)propylamine **67** (1.05 g, 6.60 mmol) and carboethoxyphthalimide (1.45 g, 6.60 mmol) in acetonitrile (10 mL) at room temperature was added triethylamine (0.92 mL, 6.60 mmol) in one portion, and the resulting mixture was stirred at room temperature for 18 h and evaporated in vacuo, and the residue was dissolved in ethyl acetate (50 mL). The solution was washed with brine, 10% citric acid solution, and brine and then dried over anhydrous magnesium sulfate. Filtration and concentration in vacuo gave an oil, which was purified by column chromatography on silica, with ethyl acetate−petroleum ether mixture as eluent, to afford a colorless oil (1.65 g). To a stirred solution of the above oil in THF (20 mL) at room temperature was added 2 N HCl solution (15 mL, 30 mmol) in one portion, and the resulting mixture was stirred at room temperature for 2 h and then evaporated in vacuo to half the initial volume. The residue was saturated with NaCl and extracted with ethyl acetate. The organic phase was dried by anhydrous magnesium sulfate. Filtration and concentration in vacuo afforded a colorless oil (1.28 g, 77%).

2-[5-((*tert*-Butyldimethylsilanyl)oxy)-4-hydroxypentyl]isoindole-1,3-dione 69. Prepared from diol **68** by general procedure for Boc derivatives; yield 91%.

2-[5-((*tert*-Butyldimethylsilanyl)oxy)-4-oxopentyl]isoindole-1,3-dione 70. Prepared from compound **69** by general procedure for Boc derivatives; yield 92%.

(*S*)-2-{3-[3-(5,7-Difluoro-1,2,3,4-tetrahydronaphthalen-2-yl)-2-thioxo-2,3-dihydro-1*H*-imidazol-4-yl]propyl}isoindole-1,3-dione 71 (General Procedure). A stirred mixture of (*S*)-5,7-difluoro-1,2,3,4-tetrahydronaphthalen-2-ylamine hydrochloride **10f** (0.22 g, 1.0 mmol), 2-[5-((*tert*-butyldimethylsilanyl)oxy)-4-oxopentyl]isoindole-1,3-dione **70** (0.38 g, 1.05 mmol), potassium thiocyanate (0.11 g, 1.10 mmol), water (0.18 g, 1.0 mmol), and acetic acid (0.3 mL, 5.0 mmol) in ethyl acetate (3 mL) was refluxed for 7 h, then cooled to room temperature, washed by sodium bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated in vacuo. The residue was purified by column chromatography on silica, with ethyl acetate−petroleum ether mixture as eluent, to afford a yellow oil (0.18 g, 40%).

(*S*)-5-(3-aminopropyl)-1-(5,7-difluoro-1,2,3,4-tetrahydronaphthalen-2-yl)-1,3-dihydroimidazole-2-thione Hydrochloride 73 (General Procedure). Compound **71** (0.16 g, 0.35 mmol) was dissolved in a mixture of 2-propanol (5 mL) and THF (2 mL). Water (0.8 mL) and sodium borohydride (0.066 g, 1.74 mmol) were added at room temperature, and the mixture was stirred for 15 h. Acetic acid (0.6 mL, 10 mmol) was added, and and the solution was refluxed for 2 h and then evaporated in vacuo to dryness. The residue was taken up into acetone, the solid was filtered off, and the filtrate was acidified with 2 N HCl solution in ethyl acetate. The precipitate was collected and washed with acetone to afford beige crystals (0.076 g, 60%): δ_H (400 MHz, DMSO-d_6) 12.1 (1H, br s, NH), 8.40 (3H, br s, N^+H), 7.05 (1H, t, C6H), 6.91 (1H, t, C8H), 6.76 (1H, s, C4′H), 4.60 (2H, br s, C1H and C2H), 3.00−2.75 (8H, m, C1H, C3H, C4H, C1″H and C3″H), 2.00 (1H, m, C3H), 1.80 (2H, m, C2″H); δ_C (100 MHz, DMSO-d_6) 161.3 (CS), 160.2 (dd, C7), 160.2 (dd, C5), 139.4 (m, C8a), 127.8 (C5′), 119.1 (dd, C5a), 115.3 (C4′), 111.0 (dd, C5), 101.3 (t, C6), 52.4 (C2), 38.0 (C3″), 31.9 (C1), 25.4 (C3), 25.1 (C1″), 21.8 (C4), 21.2 (C2″). Anal. ($C_{16}H_{20}ClF_2N_3S\cdot 0.33CH_3CO_2C_2H_5$) C, H, N, S.

Pharmacology. SK-N-SH cells (ATCC HTB-11, passages 55−79) were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Cells were grown in Eagle's minimum essential medium (Sigma−Aldrich) supplemented with 100 units/mL penicillin G, 0.25 μg/mL amphotericin B, 100 μg/mL streptomycin, 10% fetal bovine serum (Sigma−Aldrich), and 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes). For subculturing, cells were dissociated with 0.05% trypsin−EDTA, split 1:4, and subcultured in either 21 cm^2 dishes or 24-well plates (1.9 cm^2). Cell medium was changed every 2 days, and cells reached confluence 3−5 days after seeding.

Experiments were performed 7 days after seeding, and each square centimeter contained 103−200 μg of cellular protein. For 3 h prior to the experiment, the fetal bovine serum was removed from cell medium.

DBH activity was determined with dopamine as substrate and measuring noradrenaline formed by a modification of the published method.[38] In brief: the reaction mixture (total volume 500 μL) contained sodium acetate pH 5.0 (200 mM), *N*-ethylmaleimide (30 mM), $CuSO_4$ (5 μM), catalase aqueous solution (0.5 mg/ mL), pargyline hydrochloride (1 mM), sodium fumarate (10 mM), ascorbic acid (20 mM), inhibitor (5 μM) or control solution, and dopamine (50 mM prepared in 20 mM ascorbic acid). After a preincubation period in reaction mixture for 20 min at 37 °C, the reaction was initiated by the addition of dopamine and proceeded for 45 min at 37 °C. Reaction was terminated with the addition of 50 μL of 2 M perchloric acid, and plates were kept at 4 °C for 1 h, after which solutions were removed and filtered through Spin-X filter tubes of 0.22 μm pore size (Corning-Costar). Noradrenaline was then assayed in the filtrates by means of high-pressure liquid chromatography with electrochemical detection. The mobile phase was a degassed solution of citric acid (0.1 mM), sodium acetate (0.1 M), sodium octylsulfate (1 mM), EDTA (0.15 mM), dibutylamine (1 mM), and methanol (5%) adjusted to pH 3.5 with perchloric acid. The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode, and an amperometric detector (Gilson model 142); the detector cell was operated at 0.75 V. Current produced was monitored with the Gilson Unipoint LC system software.

Male NMRI mice or Wistar rats were obtained from Interfauna Ibérica (Spain) and were kept 10 and 5 per cage, respectively, under controlled environmental conditions (12 h light/dark cycle and room temperature 22 ± 1 °C). Food and tap water were allowed ad libitum, and experimentation was performed during daylight hours. At time = 0 h, animals were administered with either test compounds at a given dose or vehicle (water) delivered orally via gavage. At 2, 6, 9, 12, 18, and 24 h postdose, the animals were sacrificed by decapitation and heart (left atrium and left ventricle) and brain (frontal and parietal cortex) were isolated, weighed, and stored in a volume of 0.2 M perchloric acid for 12 h at 4 °C in the dark. Postincubation, the resulting supernatants were collected by centrifuge filtration of incubates (0.2 μM, 10 min, ∼5000 rpm, 4 °C). Supernatants were stored frozen at −80 °C until analysis. Quantification of dopamine and noradrenaline in supernatants was performed by high-pressure liquid chromatography with electrochemical detection as described above. All animal interventions were performed in accordance with European Directive 86/609 and the rules of the National Institute of Health's Guide for the Care and Use of Laboratory Animals (http://oacu.od.hin.gov/regs/guide/index.htm).

Supporting Information Available: Full NMR and combustion analysis data for target compounds not included in Experimental Section and NMR data for selected intermediate compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Parmley, W. W. Neuroendocrine changes in heart failure and their clinical relevance. *Clin. Cardiol*. **1995**, *18*, 440−445.
(2) Levine, T. B.; Francis, G. S.; Goldsmith, S. R.; Simon, A. B.; Cohn, J. N. Activity of the sympathetic nervous system and renin−angiotensin system assessed by plasma hormone levels and their relation to hemodynamic abnormalities in congestive heart failure. *Am. J. Cardiol.* **1982**, *49*, 1659−1666.
(3) Leimbach, W. N., Jr.; Wallin, B. G.; Victor, R. G.; Aylward, P. E.; Sundlof, G.; Mark, A. L. Direct evidence from intraneural recordings for increased central sympathetic outflow in patients with heart failure. *Circulation* **1986**, *73*, 913−919.
(4) Hasking, G. J.; Esler, M. D.; Jennings, G. L.; Burton, D.; Johns, J. A.; Korner, P. I. Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. *Circulation* **1986**, *73*, 615−621.
(5) Cohn, J. N.; Levine, T. B.; Olivari, M. T.; Garberg, V.; Lura, D.; Francis, G. S.; Simon, A. B.; Rector, T. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N. Engl. J. Med.* **1984**, *311*, 819−823.
(6) Benedict, C. R.; Shelton, B.; Johnstone, D. E.; Francis, G.; Greenberg, B.; Konstam, M.; Probstfield, J. L.; Yusuf, S. Prognostic significance of plasma norepinephrine in patients with asymptomatic left ventricular dysfunction. SOLVD Investigators. *Circulation* **1996**, *94*, 690−697.
(7) Pfeffer, M. A.; Stevenson, L. W. Beta-adrenergic blockers and survival in heart failure. *N. Engl. J. Med.* **1996**, *334*, 1396−1397.
(8) Goldstein, M.; Anagnoste, B.; Lauber, E.; McKereghan, M. R. Inhibition of dopamine-β-hydroxylase by disulfiram. *Life Sci*. **1964**, *3*, 763−767.
(9) Lippmann, W.; Lloyde, K. Dopamine-β-hydroxylase inhibition by dimethyldithiocarbamate and related compounds. *Biochem. Pharmacol*. **1969**, *18*, 2507−2516.
(10) Corrodi, H.; Fuxe, K.; Hamberger, B.; Ljungdahl, A. Studies on the central and peripheral noradrenaline neurons using a new dopamine-β-hydroxylase inhibitor. *Eur. J. Pharmacol.* **1970**, *12*, 145−155.
(11) Hidaka, H. Fusaric (5-butylpicolinic) acid, an inhibitor of dopamine-β-hydroxylase, affects serotonin and noradrenaline. *Nature* **1971**, *231*, 54−55.
(12) Johnson, G. A.; Boukma, S. J.; Kim, E. G. In vivo inhibition of dopamine-β-hydroxylase by 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624). *J. Pharmacol. Exp. Ther*. **1970**, *171*, 80−87.

(13) Claxton, I. M.; Palfreyman, M. G.; Poyser, R. H.; Whiting, R. L. BRL 8242 (2-[2-benzimidazolyl]-amino-2-imidazoline dihydrochloride), a new inhibitor of dopamine-β-hydroxylase with antihypertensive activity. *Eur. J. Pharmacol.* **1976**, *37*, 179−188.
(14) Kruse, L. L.; Kaiser, C.; DeWolf, W. E., Jr.; Frazee, J. S.; Erickson, R. W.; Ezekiel, M.; Ohlstein, E. H.; Ruffolo, R. R., Jr.; Berkowitz, B. A. Substituted 1-benzimidazole-2-thiols as potent and orally active inhibitors of dopamine-β-hydroxylase. *J. Med. Chem.* **1986**, *29*, 887−889.
(15) McCarthy, J. R.; Matthews, D. P.; Broersma, R. J.; McDermott, R. D.; Kastner, P. R.; Hornsperger, J.-M.; Demeter, D. A.; Weintraub, H. J. R.; Whitten, J. P. 1-(Thienylalkyl)imidazole-2(3*H*)-thiones as potent competitive inhibitors of dopamine-β-hydroxylase. *J. Med. Chem.* **1990**, *33*, 1866−1873.
(16) Martinez, G. R.; Gooding, O. W.; Repke, D. B.; Teitelbaum, P. J.; Walker, K. A. M.; Whiting, R. L. U.S. Patent 5,538,988, 1996.
(17) Stanley, W. C.; Li, B.; Bonhaus, D. W.; Johnson, L. G.; Lee, K.; Porter, S.; Walker, K.; Martinez, G.; Eglen, R. M.; Whiting, R. L.; Hedge, S. S. Catecholamine modulatory effect of nepicastat (RS-25560-197), a novel, potent and selective inhibitor of dopamine-β-hydroxylase. *Br. J. Pharmacol*. **1997**, *121*, 1803−1809.
(18) Benedict, C. R.; Gottlieb, S. S.; Massie, B. M.; Ghali, J. K.; McKinnon, S. A.; Friday, K. J. Modulation of sympathetic activation in patients with heart failure by nepicastat (RS-25560-197): a dopamine-β-hydroxylase inhibitor. Abstracts of 70th Scientific Sessions of the American Heart Association, Orlando, FL, November 9−12, 1997; *Circulation* **1997**, *96* (8S), 509.
(19) Nakano, T.; Kobayashi, K.; Saito, S.; Fujita, K.; Nagatsu, T. Mouse dopamine β-hydroxylase: primary stucture deduced from the cDNA sequence and exon/intron organization of the gene. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 590−599.
(20) McMahon, A.; Geertman, R.; Sabban, E. L. Rat dopamine β-hydroxylase: molecular cloning and characterization of the cDNA and regulation of the mRNA by resperine. *J. Neurosci. Res*. **1990**, *25*, 395−404.
(21) Wang, N.; Southan, C.; DeWolf, W. E., Jr.; Wells, T. N.; Kruse, L. I.; Leatherbarrow, R. J. Bovine dopamine β-hydroxylase, primary structure determined by cDNA cloning and amino acid sequencing. *Biochemistry* **1990**, *29*, 6466−6474.
(22) Wu, H. J.; Parmer, R. J.; Koop, A. H.; Rozansky, D. J.; O'Connor, D. T. Molecular cloning, structure, and expression of dopamine β-hydroxylase from bovine adrenal medulla. *J. Neurochem.* **1990**, *55*, 97−105.
(23) Robertson, J. G.; Desai, P. R.; Kumar, A.; Farrington, G. K.; Fitzpatrick, P. F.; Villafranca, J. J. Primary amino acid sequence of bovine dopamine β-hydroxylase. *J. Biol. Chem*. **1990**, *265*, 1029−1035.
(24) Lewis, E. J.; Allison, S.; Fader, D.; Claflin, V.; Baizer, L. Bovine dopamine β-hydroxylase cDNA. Complete coding sequence and expression in mammalian cells with vaccinia virus vector. *J. Biol. Chem.* **1990**, *265*, 1021−1028.
(25) Kobayashi, K.; Kurosawa, Y.; Fujita, K.; Nagatsu, T. Human dopamine β-hydroxylase gene: two mRNA types having different 3′-terminal regions are produced through alternative polyadenylation. *Nucleic Acids Res.* **1989**, *17*, 1089−1102.
(26) Lamouroux, A.; Vigny, A.; Faucon Biguet, N.; Darmon, M. C.; Franck, R.; Henry, J. P.; Mallet, J. The primary structure of human dopamine β-hydroxylase: insights into the relationship between the soluble and the membrane-bound forms of the enzyme. *EMBO J.* **l987**, *6*, 3931−*3937*.
(27) Kruse, L. I.; Kaiser, C.; DeWolf, W. E., Jr.; Frazee, J. S.; Ross, S. T.; Wawro, J.; Wise, M.; Flaim, K. E.; Sawyer, J. L.; Erickson, R. W.; Ezekiel, M.; Ohlstein, E. H.; Berkowitz, B. A. Multisubstrate inhibitors of dopamine β-hydroxylase. 2. Structure−activity relationships at the phenethylamine binding site. *J. Med. Chem.* **1987**, *30*, 486-494.
(28) Demeter, D. A.; Weintraub, H. J. R.; Knittel, J. J. The local minima method (LMM) of pharmacophore determination: a protocol for predicting the bioactive conformation of small, conformationally flexible molecules. *J. Chem. Inf. Comput. Sci*. **1998**, *38*, 1125−1136.
(29) Trzhtsinskaya, B. V.; Abramova, N. D. Imidazole-2-thiones: synthesis, structure, properties. *Sulfur Rep.* **1991**, *10*, 389−430.
(30) Wolf, E.; Kennedy, I. A.; Himmeldirk, K.; Spenser, I. D. 5-Hydroxypentane-2,3-dione and 3-amino-1-hydroxypropan-2-one, putative precursors of vitamin B_6. *Can. J. Chem*. **1997**, *75*, 942−948.
(31) Shiba, T.; Akiyama, H.; Umeda, I.; Okada, S.; Wakamiya, T. Synthesis and stereochemistry of hypusine, a new amino acid in bovine brain. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 899−903.
(32) Olsen, R. K.; Bhat, K. L.; Wardle, R. B.; Hennen, W. J.; Kini, G. D. Synthesis of (*S*)-(−)-3-piperidinol from L-glutamic acid and (*S*)-malic acid. *J. Org. Chem.* **1985**, *50*, 896−899.
(33) Sarda, N.; Grouiller, A.; Pacheco, H. Nouvelle synthese de l′amino-3 chromanne. Synthese et configuration absolue de ses enantiomeres. *Tetrahedron Lett.* **1976**, 271−272.
(34) Brisander, M.; Caldirola, P.; Johansson, A. M.; Hacksell, U. Alkylation of tricarbonylchromium-stabilized benzylic anions of 3-(dipropylamino)chroman. *J. Org. Chem.* **1998**, *63*, 5362−5367.
(35) Neirabeyeh, M. Al; Reynaud, D.; Podona, T.; Ou, L.; Perdicakis, C.; Coudert, G.; Guillaumet, G.; Pichat, L.; Gharib, A.; Sarda, N. Methoxy and hydroxy derivatives of 3,4-dihydro-3-(di-*n*-propylamino)-2*H*-1-benzopyrans: new synthesis and dopaminergic activity. *Eur. J. Med. Chem*. **1991**, *26*, 497−504.
(36) Eriksson, M. PCT Int. Appl. WO9959988, 1999.
(37) Napoletano, M.; Cavalleri, P.; Fraire, C.; Grancini, G. C.; Montanari, S.; Santangelo, F. PCT Int. Appl. WO9608489A1, 1996.
(38) Matsui, H.; Kato, T.; Yamamoto, C.; Fujita, K.; Nagatsu, T. Highly sensitive assay for dopamine β-hydroxylase activity in human cerebrospinal fluid by high performance liquid chromatography−electrochemical detection: properties of the enzyme. *J. Neurochem.* **1981**, *37*, 289−296.

JM051051F