Conformationally Restrained Analogs of Sympathomimetic Catecholamines. Synthesis, Conformational Analysis, and Adrenergic Activity of Isochroman Derivatives[1]

Bruno Macchia,*,† Aldo Balsamo,† Maria Cristina Breschi,‡ Grazia Chiellini,‡ Annalina Lapucci,† Marco Macchia,† Clementina Manera,† Adriano Martinelli,† Claudia Martini,‡ Roberta Scatizzi,‡ and Gloria Uccello Barretta§

Istituto di Chimica Farmaceutica e Tossicologica, Istituto Policattedra di Discipline Biologiche, Università di Pisa, 56100 Pisa, Italy, and Centro di Studio del CNR per le Molecole Stereoordinate ed Otticamente Attive, 56100 Pisa, Italy

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In previous papers dealing with the study of the conformations and the biopharmacological activity of conformationally restrained analogs of sympathomimetic catecholamines (NE and ISO), proposals were advanced for the three-dimensional molecular models A, B, and C; these models provided information about the steric requirements for the direct activation of α_1, α_2, β_1 and β_2 adrenoceptors, respectively. The 1-(aminomethyl)-6,7-dihydroxyisochromans 11 and 12 and the 1-(aminomethyl)-5,6-dihydroxyisochromans 13 and 14 (1-AMDICs) are two different types of semirigid analogs of NE and ISO. The α_1, α_2, β_1, and β_2 adrenergic properties of the 1-AMDICs 11–14 were evaluated in vitro, both by radioligand binding assays and by functional tests on isolated preparations, and were compared with those of their parent compounds (NE and ISO). The results of a conformational study carried out by means of both ^{1}H NMR spectrometry and theoretical calculations indicated that, in these 1-AMDICs, the presumed active groups (aryl moiety, amine nitrogen and benzylic ethereal oxygen) are in a spatial relationship corresponding to the one found for NE and ISO in their preferred conformations, which also proved to be the pharmacophoric conformation in the models A–C. By means of a comparison of the stereostructures of the 1-AMDICs 11–14 with their biopharmacological properties, it was possible to obtain a further definition of the model B with respect to the activation of the α_2 adrenoceptors; the superimposition of the 1-AMDICs 11 and 12 with the molecular model C made it possible to detect an area of the β-adrenergic receptors which might hinder the fit of adrenergic drugs that are analogs of catecholamines with these receptors.

Natural catecholamines act as neurotransmitters, and interact with various subtypes of adrenergic receptors to induce specific biological responses.

Natural catecholamines and drugs structurally related to them (Figure 1) are conformationally mobile molecules and can thus assume various conformations by rotation around the simple bonds C_α–C_1, C_1–C_2, and C_2–N.

A knowledge of the active conformation of catecholamines, and therefore of the spatial relationship of their presumed active groups (aryl moiety, amine nitrogen, and alcoholic hydroxyl)[3] is basic for the rational programming of new molecules capable of interacting with receptor sites. Furthermore, a knowledge of the pharmacophoric conformation of these mediators and of drugs structurally related to them offers a valid instrument to arrive at a definition of the topography of the adrenergic receptor.

Various studies have dealt with the problem of the pharmacophoric conformation of adrenergic drugs.

One of the methods most frequently used to study the correlation between the molecular conformation and the activity of drugs which present a freedom of rotation around one or more single bonds consists of the synthesis and the pharmacological study of analogs whose presumed active groups are inserted into rigid or semirigid structures.[4]

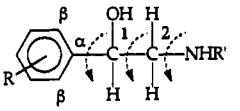

Figure 1. General formula of catecholamines and drugs structurally related to them showing the conformational freedom around the C(α)–C(1), C(1)–C(2), and C(2)–N bonds.

Previous papers took into consideration the conformationally restricted analogs of norepinephrine (NE, 1) and isoproterenol (ISO, 2) with a morpholinic (2-DPMs, 3 and 4), piperidinic (3-DPPs, 5 and 6), and tetrahydronaphthalenic (2-ADTNs, 7–10)[5] structure. Compounds 3 and 4 and 5 and 6 represent two different ways in which the C_1–C_2 portion of the side chain of amino alcohols 1 and 2 is incorporated into a cyclic structure. In tetrahydronaphthalenic derivatives 7–10, on the contrary, the conformational freedom is restricted not only around the C_1–C_2 bond but also around the C_α–C_1 one.

Comparison of the stereostructures and biopharmacological data of the semirigid analogs 3–10 with those of the corresponding open-chain compounds 1 and 2 yielded information about the conformation–activity relationship in catecholamines; it was thus possible to propose the steric models A, B, and C (see Figure 2) for interaction with the α_1, α_2, and β adrenergic receptors, respectively; these models are the result of the superimposition of the biologically most active molecules in the conformations which allow the spatial coincidence of the pharmacophoric groups.[5b]

It is possible to find in the literature the structures of certain semirigid compounds which, when arranged in

† Istituto di Chimica Farmaceutica e Tossicologica, Università di Pisa.
‡ Istituto Policattedra di Discipline Biologiche, Università di Pisa.
§ Centro di Studio del CNR per le Molecole Stereoordinate ed Otticamente Attive.
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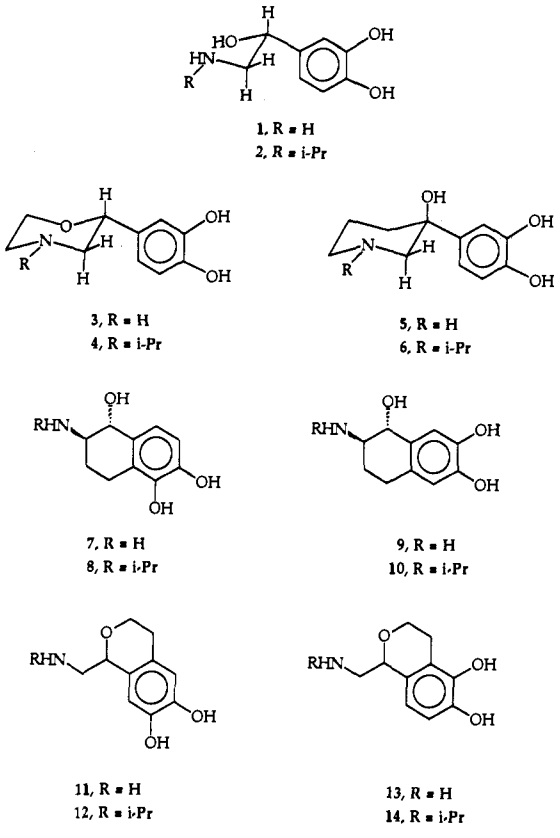


allowed conformations, may present their active groups in a spatial relationship that is practically identical to the one found in at least one of the steric models **A–C**.[6] When the pharmacological data given for these compounds are compared with those obtained for the compounds used for the construction of the models, they are found to be in agreement with the steric requirements exhibited by the models. However, for certain compounds, the pharmacological data reported are purely indicative, and no adequate conformational studies are presented.

The 1-(aminomethyl)-6,7-dihydroxyisochromans **11** and **12** and the 1-(aminomethyl)-5,6-dihydroxyisochromans **13** and **14** (1-AMDICs)[6e] represent a kind of conformationally restricted analog of NE and ISO in which the freedom of rotation around the C_α–C_1 bond is restricted. It has been reported[6e] that compound **13** possesses a good α_1 stimulating activity on the basis of functional tests carried out on a different preparation (rabbit aorta strip) from the one used by us (rat vas deferens) for this receptor. This same compound (**13**) is also described,[6e] on the basis of functional tests on guinea pig ear (β_1) or tracheal chain (β_2), as a weak β_1 antagonist, which is inactive on β_2 receptors. When its *N*-isopropyl derivative **14** was assayed by means of the same functional tests for α_1, β_1, and β_2 adrenergic receptors, it revealed a weak stimulant activity on β_2 adrenergic receptors.[6e]

Compound **12** is described[6e] as a weak β_1 antagonist, which is devoid of any β_2 adrenergic activity. Its N-unsubstituted derivative (**11**) is described as a weak stimulant of α_1 adrenergic receptors.[6e]

However, the activity of these compounds (**11–14**) had never previously been quantified numerically, nor had affinity data based on binding tests ever been published.

The aim of the present work was to synthesize the isochroman derivatives **11–14**, in order to determine their preferred conformation in solution by means of NMR studies, and to evaluate their activity on α_1, α_2, β_1, and β_2 adrenergic receptors by means of functional tests and their affinity for the same receptors by means of binding tests. The conformational study of these compounds (**11–14**) was also carried out in the gaseous state by means of theoretical studies on isolated molecules.

The availability of all these data made it possible to obtain further information about the stereostructural requirements of adrenergic receptors, by means of a further definition of the steric models **A–C** previously described.

Chemistry

The 1-AMDICs **11–14** were obtained by partially modifying the synthetic route previously described[6e,7] (see Scheme I). The 2,3-dimethoxyphenethyl alcohol (**18**) used for the synthesis of **20** was prepared starting from 2,3-dimethoxybenzaldehyde (**15**). Treatment of **15** with trimethylsulfonium iodide in the presence of a base yielded (2,3-dimethoxyphenyl)oxirane (**16**) which by anti-Markovnikov reduction with borane in the presence of boron trifluoride was transformed into the corresponding alcohol **18**. Condensation of commercially available 1,2-dimethoxy (**17**) and 2,3-dimethoxy (**18**) substituted alcohols with aminocetaldehyde diethyl acetal in the presence of dry HCl yielded the 1-(aminomethyl)-6,7- and 1-(aminomethyl)-5,6-dimethoxyisochromans (**19** and **20**), respectively.[7] The 6,7- and 5,6-dimethoxy-substituted (**21** and **22**, respectively) 1-[(isopropylamino)methyl]isochromans were obtained by reductive alkylation with acetone and sodium cyanoborohydride of the corresponding 1-(aminomethyl)isochromans **19** and **20**.

Radioligand Binding Assays

α-Adrenergic Affinity. The affinity of the 1-AMDICs **11–14** for α-adrenergic receptors was determined by binding tests carried out on rat brain membrane preparations (Table I). [^{3}H]Prazosin and [^{3}H]rauwolscine were used as specific tritiated ligands for α_1 and α_2 receptors, respectively. The results obtained for NE and ISO were found to be in agreement with previous reports.[5b,8]

Rat Brain α_1 Receptors. The N-unsubstituted compounds **11** and **13** showed a similar inhibitory activity in the [^{3}H]prazosin labeled binding assays, which was considerably lower than that of NE. The N-isopropyl-substituted compounds **12** and **14** showed a weak affinity, similar to that of ISO.

Rat Brain α_2 Receptors. The 1-AMDIC **13** showed an affinity comparable to that of NE; the other cyclic analog of NE (**11**) presented a dramatic decrease in affinity with respect to NE. The *N*-isopropyl-substituted compounds **12** and **14** showed an inhibitory affinity higher than that of ISO. The 1-AMDIC **14** was about 3 times more active in inhibiting [^{3}H]rauwolscine binding than its isomer **12**.

The K_i values shown in Table I indicate that the compounds examined interact more selectively on α_2 than on α_1 rat brain adrenoceptors.

β-Adrenergic Affinity. The β-adrenergic affinity of 1-AMDICs **11–14** (see Table I) was checked by binding tests on rat brain membranes for β_1 receptors and on bovine lung membranes for β_2 receptors. [^{3}H]CGP 26505 was used as a specific tritiated ligand for rat brain β_1 receptors. [^{3}H]DHA was used to label bovine lung β_2 receptors in the presence of 50 nM CGP 26505, which displaced [^{3}H]DHA binding from the β_1 adrenoceptor subpopulation, which represented 17% in the bovine lung. The results obtained

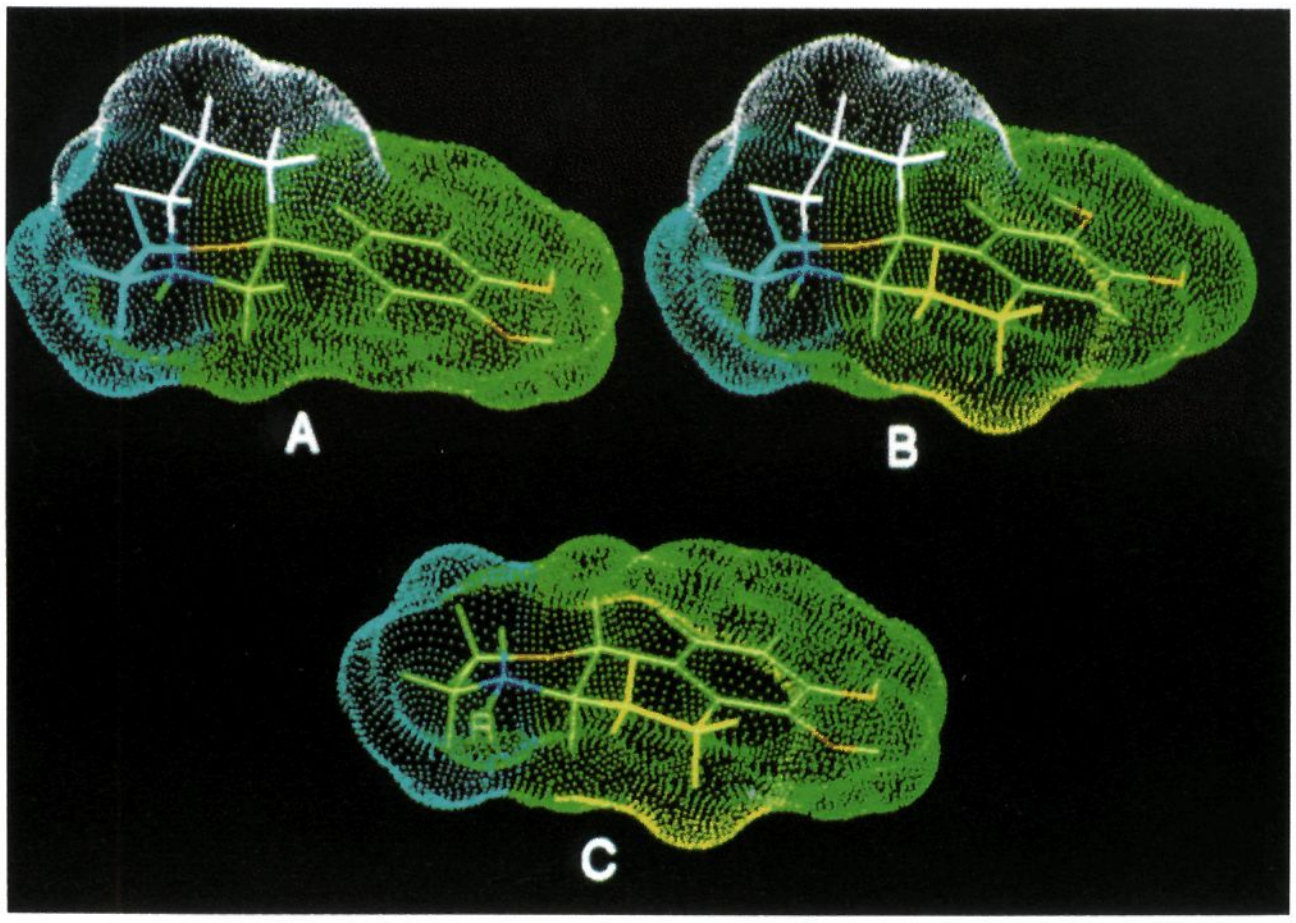
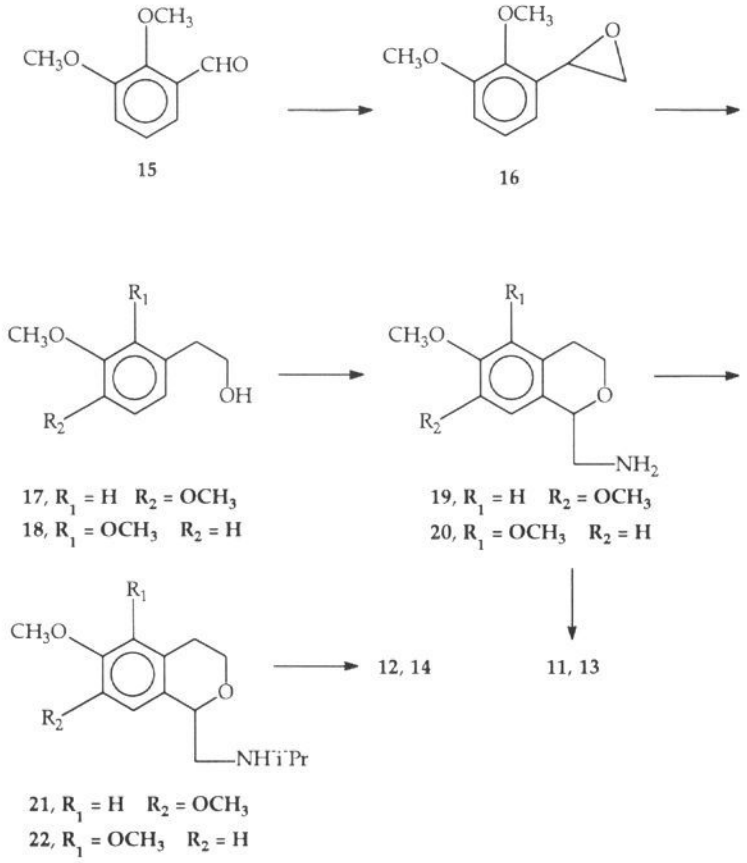


Figure 2. Molecular models arising from the superimposition of the pharmacophoric groups (aryl moiety, aminic nitrogen, alcoholic or ethereal oxygen) of drugs **1–10** in the conformations in which they should interact with the adrenergic receptors. The common arylethanolaminic portion is colored green as in NE and ISO; the other portions are colored depending on which drug (**3–10**) they arise from (the 2-DPMs **3** and **4** in cyan, the 3-DPPs **5** and **6** in white, and the 2-ADTNs **7–10** in yellow); nitrogen and oxygen atoms are blue and red, respectively. The dot clouds indicate the molecular volumes; these volumes correspond to steric hindrances that arise from atoms present in drugs **1–10** used in turn for the construction of the models and are colored depending on which drug they are generated from. These regions of bulk should not therefore hinder a hypothetical "fit" of the models with the receptor.

Scheme I



for NE and ISO were found to be in agreement with previous reports.[5b,9]

Rat Brain β_1 Receptors. K_i values of 1-AMDICs **11–14** were higher than those of NE and ISO. The *N*-isopropyl-substituted compounds (**12** and **14**) were more active than the corresponding N-unsubstituted ones (**11** and **13**). The 1-AMDICs **11** and **12** showed an affinity higher than that of the corresponding isomers **13** and **14**.

Bovine Lung β_2 Receptors. The N-unsubstituted compounds **11** and **13** exhibited comparable K_i values which were higher than that of NE. A decrease in K_i was observed on passing to the *N*-isopropyl-substituted derivatives **12** and **14**, whose affinity, however, was found to be lower than that of ISO.

Also in this case, the 1-AMDICs **11** and **12** showed an affinity higher than that of the corresponding isomers **13** and **14**.

Functional Tests

α-Adrenergic Activity. The 1-AMDICs **11–14** were tested on isolated rat vas deferens for their activity on α_1 receptors and on isolated guinea pig ileum for their activity on α_2 receptors (Table II).

Rat Vas Deferens α_1 Receptors. The 1-AMDIC **13** was found to induce a considerable dose-related contraction of the vas deferens smooth musculature; its pD_2 value was found to be 4.69 ± 0.10, which was slightly lower than that of NE on the same preparation. Also the intrinsic activity of **13** was quite similar to that of the standard agonist (NE). The 1-AMDIC **11** showed an analogous stimulating activity but with a lower potency and a very limited intrinsic activity. The 1-AMDIC **12** was devoid of any activity; **14** proved to possess a potency quite similar to that of **11**, but as an antagonist. These results are partially in contrast with the findings of Kumar et al.,[6e] who found that **12** possessed an antagonistic effect, albeit weak, on rabbit aorta strips and that **14** had a hypertensive effect when injected into anaesthetized cats. The stimulant effects observed for **11** and **13** were produced by a direct mechanism, because some additional tests performed on tissues from reserpine-pretreated rats gave practically identical results to those obtained with untreated animals.

Guinea Pig Ileum α_2 Receptors. All the 1-AMDICs tested (**11–14**) showed a considerable stimulant activity on α_2 presynaptic receptors. **13** was the most active, and

Table I. Radioligand Adrenergic Binding Affinities of Compounds 1, 2, and 11–14

	K_i, nM[a]			
	α adrenergic binding affinity		β adrenergic binding affinity	
compd	rat brain (α_1)	rat brain (α_2)	rat brain (β_1)	bovine lung (β_2)
1	450 (390–520)	4.8 (4.5–7.1)	126 (108–144)	6000 (5200–6600)
11	60300 (51900–69000)	1280 (1250–1320)	3450 (2850–4140)	28800 (23200–33400)
13	42200 (37100–48000)	7.4 (6.0–9.0)	40300 (30200–52400)	49900 (41800–58700)
2	35500 (32000–39500)	21000 (16000–24000)	80 (53–100)	110 (100–130)
12	26000 (19100–34800)	4120 (3200–5240)	1490 (1190–1970)	4900 (3470–6450)
14	21500 (16600–27400)	1780 (1550–2040)	28800 (24200–34200)	14900 (13300–15900)

[a] Geometric means of five separate determinations with confidence limits shown in parentheses.

Table II. Adrenergic Activities[a] of Compounds 1, 2, and 11–14 on Isolated Preparations

	α adrenergic activity[a]						β adrenergic activity[a]					
	isolated rat vas deferens (α_1)			isolated guinea pig ileum (α_2)			isolated guinea pig atria (β_1)			isolated guinea pig tracheal strip (β_2)		
compd	pD_2	ia[b]	$-\log IC_{50}$[c]	pD_2	ia[b]	$-\log IC_{50}$[c]	pD_2	ia[b]	$-\log IC_{50}$[c]	pD_2	ia[b]	$-\log IC_{50}$[c]
1	5.12(±0.10)	1.00	–	6.56(±0.11)	1.00	–	6.32(±0.07)	1.00	–	6.03(±0.06)	1.00	–
11	3.97 (±0.05)	0.42	–	5.45(±0.10)	0.73	–	–	–	5.17(±0.16)	–	–	4.52(±0.34)
13	4.69(±0.10)	1.03	–	7.68(±0.24)	1.00	–	–	–	4.18(±0.17)	–	–	<3.50
2	3.50(±0.14)	0.83	–	4.95(±0.14)	0.74	–	8.45(±0.12)	1.00	–	8.33(±0.18)	1.00	–
12	–	–	–	4.56(±0.05)	0.72	–	–	–	4.47(±0.11)	–	–	5.26(±0.05)
14	–	–	<3.50	5.26(±0.23)	1.00	–	–	–	<3.50	<3.50	–	–
propranolol							–	–	7.46(±0.10)	–	–	7.54(±0.15)

[a] The values represent the mean of 4–6 experiments for each drug ± standard error in parentheses. [b] Intrinsic activity, i.e. the ratio between the maximal response elicited by the compound under test and that elicited by the full agonist, namely NE and ISO, for α and β adrenoceptors, respectively. [c] $-\log IC_{50}$ is the negative logarithm of the concentration that reduce the agonist response by 50%.

its potency was higher than that of NE. 11 and 14 showed a good agonistic activity of the same potency, as indicated by the similar pD_2 and intrinsic activity values. Even the agonistic activity of the least active compound (12) was not negligible.

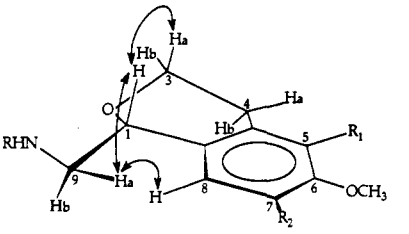
As expected, the N-unsubstituted compounds (11 and 13) proved to be more active than the corresponding *N*-isopropyl-substituted ones (12 and 14).

The pD_2 and ia values shown in Table II indicate that the 1-AMDICs 11–14 act more selectively on α_2 than on α_1 adrenoceptors.

β-Adrenergic Activity. The 1-AMDICs 11–14 were tested on isolated guinea pig atria for their activity on β_1 receptors and on isolated guinea pig tracheal strips for their activity on β_2 receptors (Table II).

Guinea Pig Atria β_1 Receptors. All the 1-AMDICs 11–14 exhibited an antagonistic action on these receptors but with various degrees of potency. 11 was the most active, followed by its *N*-isopropyl derivative (12). 13 and 14 were weak antagonists and, in analogy with the results obtained for 11 and 12, the *N*-isopropyl derivative (14) showed a $-\log IC_{50}$ value (<3.5) lower than that of the N-unsubstituted compound (13). These results are in agreement with those obtained by Kumar et al.,[6e] who defined the same compounds as weak β_1 antagonists, with the exception of 14.

Guinea Pig Tracheal Strip B_2 Receptors. On this preparation, too, the 1-AMDICs tested (11–14) revealed an antagonistic action, the only exception being 14, which, as reported by Kumar et al.,[6e] showed a very weak agonistic activity. 13 was poorly active, and its $-\log IC_{50}$ was the lowest among the compounds tested. The 1-AMDICs 11 and 12 gave $-\log IC_{50}$ values of the same order of magnitude as those obtained for the same drugs on guinea pig atria β_1 receptors, with the difference that, for tracheal β_2 receptors, the most active drug was 12. For 11, 13, and 14, our results are substantially in agreement with those reported by Kumar et al.;[6e] the good activity found by us for 1-AMDIC 12, however, does not agree with the findings of the same authors,[6e] who describe this compound as inactive on β_2 receptors.



Compd	R	R_1	R_2
19	H	H	OCH_3
20	H	OCH_3	H
21	i-Pr	H	OCH_3
22	i-Pr	OCH_3	H

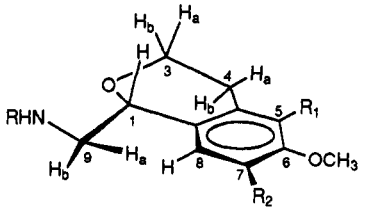
Figure 3. Diagnostic NOEs indicating the preferred conformation of compounds 19–22 in $CDCl_3$ solution.

Conformational Analysis

NMR Study. In order to obtain information about the conformational situation of the 1-AMDICs 11–14 in solution, an NMR 2D NOESY was carried out on the corresponding methoxy derivatives 19–22, chosen in view of the relatively limited stability of free catecholic nuclei in solution. This study indicated that compounds 19–22, and therefore presumably also 11–14, should exist preferentially in the conformation shown in Figure 3.

As regards the N-unsubstituted compounds (19 and 20), the 2D NOESY trace corresponding to the H(1) proton showed NOEs on the H(8), H(9a), H(9b), H(3a) protons and on the NH_2 protons. The trace corresponding to the H(3a) proton showed NOEs on the H(3b), H(1), and H(4a) protons. The trace corresponding to the NH_2 protons presented NOEs on the H(9a), H(9b), and H(1) protons; no NOEs were observed on the protons of the aromatic

Table III. ^{1}H NMR Data of Isocroman Derivatives **19–22**



	δ, multiplicity												
compd	H(4a)	H(4b)	H(9b)	H(9a)	H(3a)	5-OMe	6-OMe	7-OMe	H(3b)	H(1)	H(5)	H(7)	H(8)
19	2.56, dt (J^a = 16.0, 3.5, 3.5)	2.87, ddd (J = 16.0, 9.8, 5.3)	2.97, dd (J = 13.6, 6.8)	3.10, dd (J = 13.6, 2.9)	3.72, ddd (J = 11.2, 9.8, 3.5)		3.82, s	3.80, s	4.10, ddd (J = 11.2, 5.3, 3.5)	4.67, dd (J = 2.9, 6.8)	6.58, s		6.52, s
20	2.76, ddd (J = 15.9, 4.3, 6.0)	2.79, ddd (J = 15.9, 7.6, 4.3)	2.96, dd (J = 13.6, 7.0)	3.11, dd (J = 13.6, 3.0)	3.69, ddd (J = 11.2, 7.6, 6.0)	3.78, s	3.81, s		4.11, dt (J = 11.2, 4.3, 4.3)	4.64, dd (J = 3.0, 7.0)		6.74, d (J = 8.2)	6.77, d (J = 8.2)
21	2.63, ddd (J = 16.0, 4.0, 4.6)	2.84, ddd (J = 16.0, 8.9, 4.6)	2.86, dd (J = 12.1, 9.5)	3.07, dd (J = 12.1, 3.0)	3.77, ddd (J = 11.1, 4.0, 8.9)		3.85, s	3.84, s	4.11, dt (J = 11.1, 4.6, 4.6)	4.82, dd (J = 3.0, 9.5)	6.60, s		6.59, s
22	2.82, ddd (J = 15.9, 5.4, 4.6)	2.83, ddd (J = 15.9, 4.6, 7.9)	2.81, dd (J = 12.2, 9.5)	3.09, dd (J = 12.2, 3.0)	3.74, ddd (J = 11.4, 5.4, 7.9)	3.81, s	3.85, s		4.13, dt (J = 11.4, 4.6, 4.6)	4.81, dd (J = 3.0, 9.5)		6.78, d (J = 8.6)	6.82, d (J = 8.6)

[a] All J in hertz.

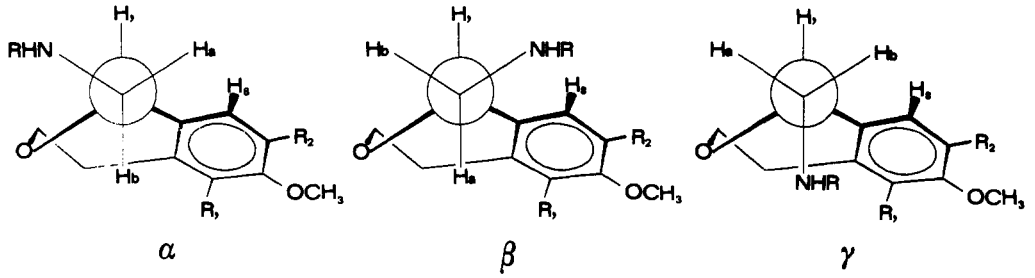


Figure 4. Newman projections of the three classical staggered rotamers around the C(1)–C(9) bond of dimethoxyisochromans **19–22**.

moiety or on the H(3) or H(4) protons of the heterocyclic ring. The trace corresponding to the aromatic protons H(5) and H(8), in the case of **19**, or H(7) and H(8), in the case of **20** (which practically coincide in the proton spectrum), showed NOEs on the H(1), H(9a), and H(9b) protons; in the case of **19**, an NOE was observed on the two methoxyls on C(6) and C(7), which are adjacent to the aromatic protons, while in the case of **20**, a single NOE was observed on the methoxyl bonded to C(6), adjacent to H(7), but not on the methoxyl in position 5, which is situated far away from both H(7) and H(8) protons. Furthermore, the traces of the aromatic protons of **19** and **20** do not reveal any NOEs on the NH_2 protons.

In the case of compounds **21** and **22**, which have an isopropyl group on the nitrogen atom, the analysis of their NOESY spectra gave results analogous to those obtained for **19** and **20**, except that the traces corresponding to the H(1) and H(8) protons did not show any NOEs on the H(9b) proton. The trace corresponding to the protons of the isopropyl group did not show any significant NOEs on the protons of their aromatic or heterocyclic moieties.

The above data suggested that, in the compounds studied (**19–22**), the aminomethyl group and the H(1) proton were, respectively, in the equatorial and axial positions. This assignment is justified by the presence of the reciprocal H(3a)–H(1) NOE and by the absence of NOEs between the protons of the aminomethyl group and the H(3) and H(4) protons of the heterocyclic ring.

As regards the conformational preference around the C_1–C_9 bond, the values of the vicinal coupling constants $J_{H(1)-H(9a)}$ and $J_{H(1)-H(9b)}$ (see Table III) suggested that the preferred conformation around the C_1–C_9 bond should correspond to the one present in one of the two rotamers α and β (see Figure 4), in which the two protons linked to C(9) are in a gauche and trans relationship with respect to H(1), respectively. Furthermore, the absence of reciprocal NOEs between the H(8) proton and the protons of the NHR group was an indication that the amino group was far from the aromatic moiety, thus establishing rotamer α (Figure 4) as the preferential one. NOEs were observed for the N-unsubstituted compounds (**19** and **20**) between protons H(8) and H(9a), and between protons H(8) and H(9b), which are possible in the α and γ rotamers, respectively. On the contrary, a single NOE is present in the *N*-isopropyl-substituted compounds (**21** and **22**) between protons H(8) and H(9a). These data indicate a greater conformational rigidity for the latter compounds (**21** and **22**) around the C(1)–C(9) bond, and therefore a more marked preference for the α conformer shown in Figure 4, in which H(9a) is close to H(8). These results are in agreement with the findings for the *N*-isopropyl-substituted compounds (**21** and **22**) that while the J_{trans} values are higher than the corresponding J values obtained for the N-unsubstituted compounds (**19** and **20**), the J_{gauche} values are substantially similar.

Theoretical Calculations. A conformational analysis was carried out on the 1-AMDICs **11–14** by means of the Discover[10] molecular mechanics program.

The two possible half-chair conformations that the dihydropyran ring can assume were taken into account, and for each of them, the conformational energy was studied as a function of the τ torsion angle (N–C–C–C, see Figure 5); all the compounds **11–14** were considered both as free bases and as cationic forms.

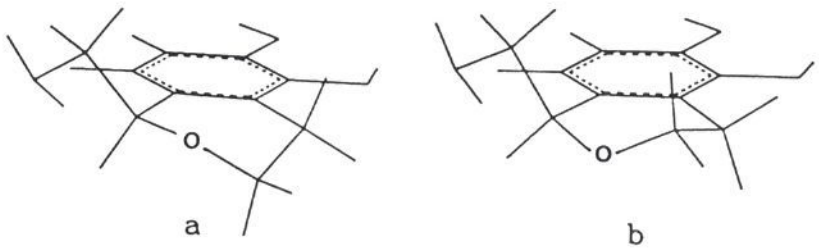


Figure 5. Compound **13** in the two possible half-chair conformations (**a** and **b**) of the dihydropyran ring.

Figure 5 shows the two half-chair conformations **a** and **b** of compound **13**, thus obtained. The energy difference between the **a** and **b** conformations shown is 0.29 kcal/mol, the **a** conformation being the preferred one (0.48 kcal/mol at *ab initio* SCF STO3-21G level). Figure 5 also shows that the CH_2NHR substituent, which is in a pseudoequatorial and a pseudoaxial position in **a** and **b**, respectively, really lies in equivalent positions, with respect to the aromatic ring, in both conformations.

Almost identical results were also found for the conformations of the other compounds **11**, **13**, and **14**, with similar energy differences between the **a** and **b** conformations.

The conformational energy trend of **11–14** was then studied as a function of the τ torsion angle (N–C–C–C). In the case of the free bases, the absolute minimum corresponds for all compounds to a value of $\tau = 90°$, whether the dihydropyran ring is in the **a** or the **b** conformation. The conformations with $\tau = 180°$ correspond to relative minima with an energy about 0.5 kcal/mol higher in **11** and **12** and about 1.6 kcal/mol higher in **13** and **14**, with respect to the conformations with $\tau = 90°$. It may be pointed out that the conformation of relative minimum **a** ($\tau = 180°$) corresponds to the preferred one found in the NMR studies.

In the case of the cationic forms, the absolute minimum was found for all compounds **11–14** with $\tau = -90°$, when the dihydropyran ring assumes the **a** conformation. Other relative minima were found with $\tau = 180°$, when the dihydrofuran ring is situated both in the **a** and in the **b** conformation, with relative energies of about 1.3 and 2.5 kcal/mol, respectively higher than the absolute minimum. The absolute minimum conformation **a** ($\tau = -90°$) was found to be stabilized by the formation of a hydrogen bond between the cationic nitrogen and the oxygen of the dihydropyran ring. The importance of this intramolecular interaction, which is considerable in isolated molecules, should be reduced in aqueous solution, or at least in polar solvents, where strong hydrogen bonds with the solvent are possible.[11]

Discussion

α-Adrenoceptor Activity. The functional test results (see Table II) indicate an activity trend that is substantially similar to the one revealed in the binding tests (Table I);[12–14] however, for the 1-AMDIC **13**, the good pD_2 value (4.69 ± 0.10) found is not confirmed by the K_i value ($42\,200 \pm 3600$) obtained in the binding tests. In addition, it may be observed that for all the 1-AMDICs **11–14**, the activity toward α_2 receptors is higher than that for α_1 receptors.

The results obtained with the 1-AMDICs **11–14** (see Tables I and II) show that a different type of limitation of the conformational freedom of the aromatic ring of NE and ISO obtained by using isochroman derivatives such as **11** and **12**, and **13** and **14**, respectively, is important for the fulfilment of the α-adrenergic activity. The cyclization which leads to the 5,6-dihydroxy derivatives **13** and **14** gives markedly better results than the one which leads to the 6,7-dihydroxy derivatives **11** and **12**; this is particularly

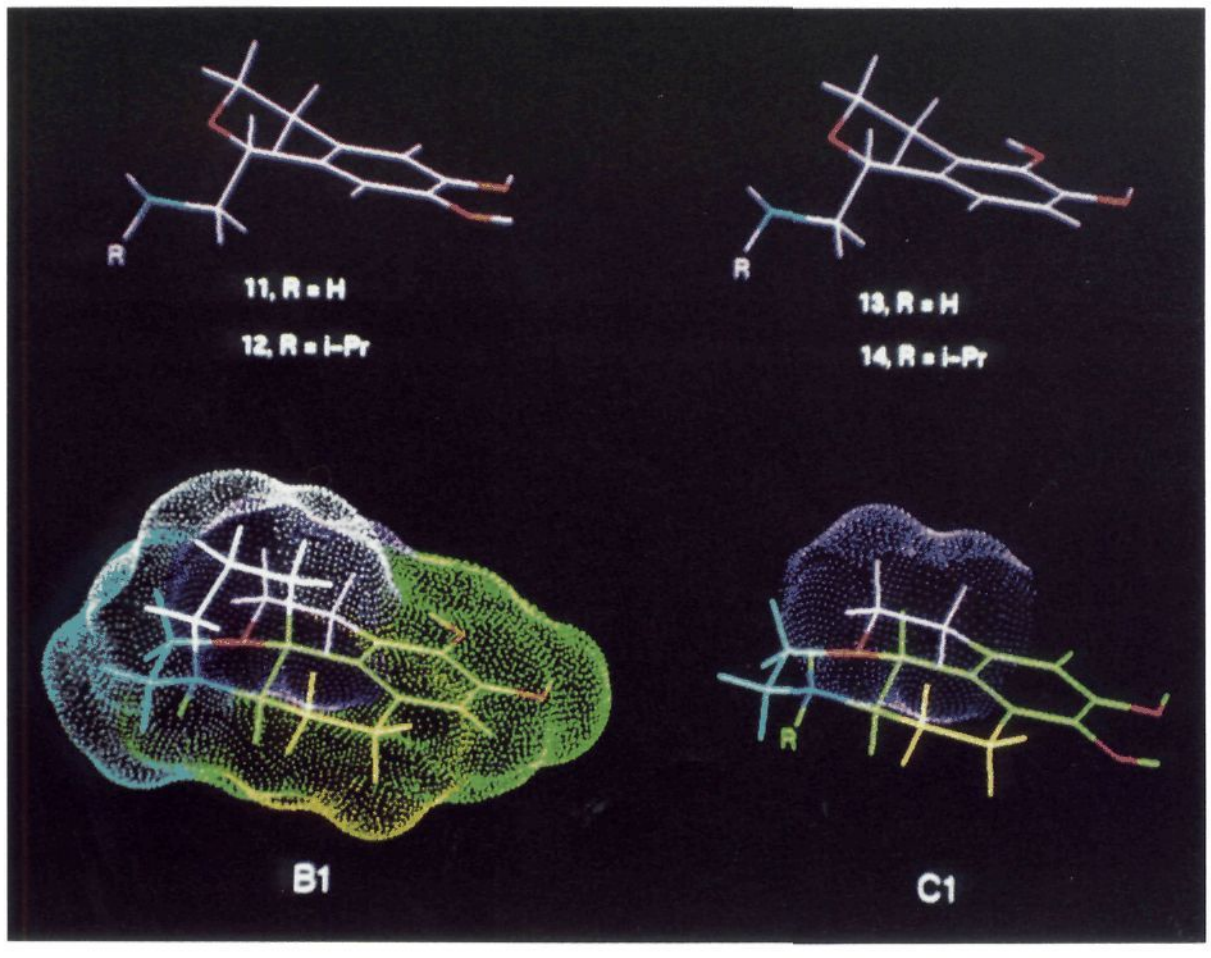


Figure 6. The 1-AMDICs **11–14** (colored in purple) in their preferred conformations. Molecular model ($\mathbf{B_1}$) arising from the superimposition of the pharmacophoric groups (aryl moiety, aminic nitrogen, and ethereal benzilic oxygen) of **13**, in the conformation in which it should interact with the α_2 adrenergic receptors, with the model **B**. The dot cloud indicates the molecular volume and it is colored depending on which drug it is generated from. This volume corresponds to steric hindrances that arise from atoms present in **13** and in model **B**. Superimposition ($\mathbf{C_1}$) of the 1-AMDCs **11** and **12** with the molecular model for the activation of the β receptors (**C**). The purple dot cloud indicates the volume of the ethylenic portion of the dihydropyran ring of **11** and **12** which may hinder a hypothetical fit with the β receptors.

evident in the case of α_2 receptors, where the activity of 13 and 14 is highly similar to that of NE and ISO, respectively.

β-Adrenoceptor Activity. Also for this type of receptor, the trend of the results of the functional tests is analogous to that of the binding tests. All the 1-AMDICs 11–14 show a lower affinity than the corresponding open-chain compounds NE and ISO, and none of them present any stimulant activity. The 1-AMDICs 11–13, on the contrary, exhibit an appreciable blocking activity. These results indicate that, while determining the disappearance of the stimulant activity, the cyclization of the catecholamines to their corresponding isochroman derivatives nevertheless permits interaction with the receptor, albeit less complete than that of the corresponding parent compounds NE and ISO.

The K_i and $-\log IC_{50}$ values indicate that, unlike findings for α receptors, the two 1-AMDICs 11 and 12 present a greater capacity to interact with both types of β receptors, compared with the isomers 13 and 14.

The different orientation of the catecholic ring in the 1-AMDICS 13 and 14, which are more active on α receptors, and in the 1-AMDICs 11 and 12, which are more active on β receptors, is in agreement with the hypothesis previously advanced about the importance of the rotameric position of the aromatic ring of catecholamines in the interaction with α- and β-adrenergic receptors.[5b,6a,15]

Molecular Models. Figure 6 shows the 1-AMDICs 11–14 in the preferential conformation found by ^1H NMR studies, which corresponds to one of the two relative minimum conformations, determined by theoretical studies. As may be observed, the 1-AMDICs in this conformation present their presumed active groups (aryl moiety, amine nitrogen, and benzylic ethereal oxygen) in a spatial situation which corresponds to that of the same groups present in NE and ISO in their preferred conformations, and in the steric models **A**–**C** (see Figure 2).[5b]

The modest activity and affinity of the 1-AMDIC 11 toward both kinds of α adrenergic receptors do not make it possible to use it to obtain a greater definition of the models **A** and **B**. The low affinity of the 1-AMDIC 13, despite the good values found for pD_2 and the intrinsic activity on α_1 receptors, does not make it possible to use this compound for the extension of the model previously proposed (**A**) for the activation of this receptor. On the contrary, the high activity and binding affinity of this same compound (13) on the α_2 receptor make it possible to use it in order to obtain a further definition of model **B**. The new model ($\mathbf{B_1}$; see Figure 6) is a result of the superimposition of 13, in its preferential conformation, on the model previously proposed (**B**). This new model ($\mathbf{B_1}$) makes it possible to determine a further molecular portion, corresponding to the ethylenic moiety of the dihydropyran ring of the isochroman system, which is not an obstacle for interaction with the α_2 receptor.

The fact that the 1-AMDIC 13 exhibits a greater affinity and activity on α_2 receptors compared with the 1-AMDIC 11 is not surprising: the isochroman derivatives 11 and 13 are analogs of NE that are conformationally restricted around the C_α–C_1 bond; in the more active isochroman derivative (13), unlike 11, the catecholic portion possesses an orientation, compared with the other active groups of the molecule, which coincides with the pharmacophoric one for α_2 receptors, determined by model **B**.[5b]

As regards β adrenergic receptors, the fact that all the 1-AMDICs 11–14 appear to show a limited affinity for these kinds of receptors, and thus either are inactive, like 14, or possess a certain β-blocking activity, like 11–13, instead of the stimulant activity typical of the corresponding open-chain compounds NE and ISO, does not make it possible to use them for the extension of model **C**, which refers to the activation of β receptors. For the 1-AMDICS 13 and 14, these results were to be expected on the basis of the observation that the catecholic system in these compounds is situated in a rotameric position which does not correspond to the one indicated as pharmacophoric by model **C**.[5b] By contrast, in the case of the 1-AMDICs 11 and 12, the catecholic system is situated in the rotameric position, which corresponds to the one indicated as pharmacophoric by model **C**. A possible explanation for the absence of the stimulating properties in 11 and 12 might be offered by an unfavorable steric effect deriving from the ethylenic group of the dihydropyran system. As shown by the superimposition of 11 and 12 on model **C** ($\mathbf{C_1}$ of Figure 6), this group comes to occupy a spatial portion which appears, in model **C** itself, to be accessible to possible receptor steric hindrances; the presence of this ethylenic group in the 1-AMIDCs 11 and 12 might therefore impede a close fit of these compounds with the β adrenergic receptors.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^1H NMR spectra of 16 and 18 were detected with a Varian EM 360 A instrument in ca. 5% solution of $CDCl_3$ using Me_4Si as the internal standard. The ^1H NMR spectra of 19–22, as hydrochlorides, and 11–14, as hydrobromides, were detected in ca. 2% D_2O solution with a Varian CTF-20 instrument operating at 80 MHz; $Me_3Si(CH_2)SO_3$-Na was used as the internal standard. The ^1H NMR and 2D-NOESY spectra of compounds 19–22 as free bases were recorded using a Varian VXR-300 spectrometer in ca. 0.08 M $CDCl_3$ solution, and the temperature was controlled to ±0.1 °C. All the solutions were accurately degassed by freeze–pump–thaw cycles. The NOESY spectra were recorded in the phase-sensitive mode. A mixing time of 800 ms was used. A spectral width of about 2500 Hz was used in both ω_1 and ω_2 dimensions. The pulse delay was maintained at 5 s; 512 hypercomplex increments of 16 scans and 2K data points each were collected. The data matrix was zero-filled at 2K × 1K and a Gaussian function was applied for processing in both dimensions. Evaporations were made in vacuo (rotating evaporator). $MgSO_4$ was always used as the drying agent. Elemental analyses were performed by our analytical laboratory and agreed with the theoretical values to within 0.4%.

(2,3-Dimethoxyphenyl)oxirane (16). A mixture of powdered sodium hydride 60% in mineral oil (2.0 g, 0.05 mol) was washed with hexane and then suspended in anhydrous DMSO (40.0 mL) and stirred under nitrogen at 75 °C until evolution of hydrogen ceased (ca. 0.5 h). The solution was cooled to room temperature, diluted with anhydrous THF (40 mL), and then cooled to −5 °C with an ice–salt bath. A solution of trimethylsulfonium iodide (10.2 g, 0.05 mol) in anhydrous DMSO (40 mL) was added to the resulting mixture over the course of about 3 min and without stirring. After completion of the addition, the reaction mixture was stirred for 1 min and then treated dropwise with a solution of 15 (6.9 g, 0.041 mol) in anhydrous DMSO (100 mL). The mixture was stirred at the same temperature for 15 min and at room temperature for 12 h and was then poured into H_2O and extracted with AcOEt. Evaporation of the washed (H_2O) and dried extracts yielded an oil consisting of 16 (6.0 g, 81%) which was directly used in the subsequent transformation. An analytical sample of pure 16 had bp 123 °C (0.6 mm); ^1H NMR δ 2.60–3.28 (m, 2H), 3.88 (s, 6H), 4.23 (t, J = 4 Hz, 1H), 6.62–7.25 (br, 3H). Anal. ($C_{10}H_{12}O_3$) C, H.

2-(2,3-Dimethoxyphenyl)ethanol (18). A stirred solution of $NaBH_4$ (1.06 g, 28.02 mmol) in anhydrous THF (7.5 mL) was

cooled in an ice bath under nitrogen and then treated with a solution of BF_3·Et_2O (3.45 mL, 28.02 mmol) in anhydrous THF (8.5 mL). The resulting mixture was diluted with anhydrous THF (65 mL) and then treated in succession with BF_3·Et_2O (3.45 mL, 28.02 mmol) and a solution of **16** (5.05 g, 28.02 mmol) in anhydrous THF (28 mL). The reaction mixture was stirred for 1 h at the same temperature and was then hydrolyzed with water and saturated with potassium carbonate. Evaporation of the washed (H_2O) and dried organic layer yielded an oil which was purified by distillation to afford **18** (2.0 g, 40%): bp 93–95 °C (0.6 mm) [lit. bp[7] 172–174 °C (22 mm)]; ^{1}H NMR δ 2.91 (t, 2H, J = 7.0 Hz), 6.71–7.23 (br, 3H).

1-(Aminomethyl)-6,7-dimethoxyisochroman hydrochloride (19·HCl) was obtained following the synthetic route previously described. **19**·HCl: mp 248–249 °C (lit. mp[7] 250–255 °C); ^{1}H NMR δ 2.66–2.96 (m, 2H), 3.38–3.54 (m, 2H), 3.85 (s, 6H), 3.95–4.31 (m, 2H), 4.94–5.18 (m, 1H), 6.82 (s, 1H), 6.91 (s, 1H).

Compound **19**·HCl was converted to the free base by treating an aqueous solution of the salt with solid KOH and extracting the free base with $CHCl_3$. The $CHCl_3$ layer was washed (H_2O), filtered, and evaporated to give pure **19** as an oil (for the ^{1}H NMR spectral data, see Table III). Anal. ($C_{12}H_{17}NO_3$) C, H, N.

1-(Aminomethyl)-5,6-dimethoxyisochroman hydrochloride (20·HCl) was obtained following the synthetic route previously described. **20**·HCl: mp 201–202 °C (lit. mp[7] 200–202 °C); ^{1}H NMR δ 2.65–2.94 (m, 2H), 3.24–3.52 (m, 2H), 3.80 (s, 3H), 3.88 (s, 3H), 3.91–4.31 (m, 2H), 4.92–5.16 (m, 1H), 7.03 (s, 2H).

Compound **20**·HCl was converted to the free base by treating an aqueous solution of the salt with solid KOH and extracting the free base with $CHCl_3$. The $CHCl_3$ layer was washed (H_2O), filtered, and evaporated to give pure **20** as an oil (for the ^{1}H NMR spectral data, see Table III). Anal. ($C_{12}H_{17}NO_3$) C, H, N.

1-(Aminomethyl)-6,7-dihydroxyisochroman hydrobromide (11·HBr) was obtained following the synthetic route previously described: mp 260–262 °C dec (MeOH–Et_2O) (lit. mp[6e] 258–260 °C dec); ^{1}H NMR δ 2.58–2.91 (m, 2H), 3.29–3.53 (m, 2H), 3.59–4.32 (m, 2H), 4.88–5.14 (m, 1H), 6.70 (s, 1H), 6.76 (s, 1H).

1-(Aminomethyl)-5,6-dihydroxyisochroman hydrobromide (13·HBr) was obtained following the synthetic route previously described: mp 248–250 °C (Me_2CO–Et_2O) (lit. mp[6e] 250–254 °C); ^{1}H NMR δ 2.59–2.84 (m, 2H), 3.48–3.52 (m, 2H), 3.59–4.32 (m, 2H), 4.89–5.15 (m, 1H), 6.67 (d, 1H, J = 8.3 Hz), 6.86 (d, 1H, J = 8.3 Hz).

1-[(Isopropylamino)methyl]-6,7-dimethoxyisochroman Hydrochloride (21·HCl). $NaBH_3CN$ (0.31 g, 4.93 mmol) was added in portions to a solution of **19** (0.54 g, 2.42 mmol) in a mixture of Me_2CO (5.0 mL) and anhydrous MeOH (24 mL) and the reaction mixture was stirred at room temperature. After 24 h, the mixture was poured into H_2O and extracted with AcOEt. Evaporation of washed (H_2O) and dried extracts yielded crude **21** as an oil (0.55 g), which was dissolved in anhydrous Et_2O and then treated with an excess of Et_2O·HCl. The solid precipitate was filtered and crystallized from MeOH–Et_2O to yield pure **21**·HCl (0.50 g, 68%): mp 190–193 °C (lit. mp[6e] 188–190 °C); ^{1}H NMR δ 1.12 (d, 6H, J = 6.5 Hz), 2.61–2.88 (m, 2H), 2.93–3.31 (m, 3H), 3.89 (s, 6H), 4.71–5.06 (m, 1H), 6.83 (s, 2H).

Compound **21**·HCl was converted to the free base by treating an aqueous solution of the salt with solid KOH and extracting the free base with $CHCl_3$. The $CHCl_3$ layer was washed (H_2O), filtered, and evaporated to give pure **21** as an oil (for the ^{1}H NMR spectral data, see Table III). Anal. ($C_{15}H_{23}NO_3$) C, H, N.

1-[(Isopropylamino)methyl]-5,6-dimethoxyisochroman (22·HCl). $NaBH_3CN$ (0.58 g, 9.23 mmol) was added portionwise at room temperature to a stirred solution of **20**·HCl (0.60 g, 2.31 mmol) in a mixture of Me_2CO (4.80 mL) and anhydrous MeOH (23 mL). After 24 h the reaction mixture was poured into H_2O and extracted with AcOEt. Evaporation of the washed (H_2O) and dried extracts yielded crude **22** as an oil (0.50 g), which was dissolved in anhydrous Et_2O and treated with an excess of Et_2O·HCl. The solid precipitate was filtered and crystallized from MeOH–Et_2O to yield pure **22**·HCl (0.45 g, 65%): mp 220–222 °C dec (lit. mp[6e] 223–225 °C dec); ^{1}H NMR δ 1.36 (d, 6H, J = 6.6 Hz), 2.73–2.99 (m, 2H), 3.35–3.64 (m, 3H), 3.81 (s, 3H), 3.89 (s, 3H), 3.96–4.46 (m, 2H), 4.98–5.26 (m, 2H), 7.04 (s, 2H).

Compound **22**·HCl was converted to the free base by treating an aqueous solution of the salt with solid KOH and extracting the free base with $CHCl_3$. The $CHCl_3$ layer was washed (H_2O), filtered, and evaporated to give pure **22** as an oil (for the ^{1}H NMR spectral data see Table III). Anal. ($C_{15}H_{23}NO_3$) C, H, N.

1-[(Isopropylamino)methyl]-6,7-dihydroxyisochroman hydrobromide (12·HBr) was obtained following the synthetic route previously described: mp 173–176 °C (lit. mp[6e] 165–170 °C); ^{1}H NMR δ 1.35 (d, 6H, J = 6.6 Hz), 2.54–2.86 (m, 2H), 3.21–3.65 (m, 3H), 3.75–4.28 (m, 2H), 4.87–5.16 (m, 1H), 6.68 (s, 1H), 6.75 (s, 1H).

1-[(Isopropylamino)methyl]-5,6-dihydroxyisochroman hydrobromide (14·HBr) was obtained following the synthetic route previously described: mp 205–208 °C (lit. mp[6e] 208–210 °C); ^{1}H NMR δ 1.30 and 1.34 (2d, 6H, J = 6.5 Hz), 2.62–2.82 (m, 2H), 3.25–3.69 (m, 3H), 3.71–4.28 (m, 2H), 4.87–5.23 (m, 1H), 6.67 (d, 1H, J = 8.2 Hz), 6.87 (d, 1H, J = 8.2 Hz).

Radioligand Binding Methods. Rat Brain α_1 and α_2 Receptors. α_1 and α_2 receptor binding were determined in rat cerebral cortex membranes as elsewhere reported.[16]

Rat Brain β_1 Receptors. β_1 receptors were assayed in rat cortical membranes following the procedure previously described.[5b]

Bovine Lung β_2 Receptors. β_2 receptor bindings were studied in bovine lung using [^{3}H]DHA (dihydroalprenolol) as the ligand (Du Pont de Nemours, New England Nuclear Division, specific activity = 48.1 Ci/mmol).

Membranes were obtained by lung homogenization in 1:20 volumes of 0.32 M sucrose, followed by centrifugation at 800g for 10 min at 5 °C. The supernatant was recentrifuged at 30000g for 10 min at 5 °C. The resulting pellet was suspended in 50 mM phosphate buffer at pH 7.4 containing 0.02% ascorbic acid and then centrifuged. This step was repeated twice. Crude lung membranes were suspended in phosphate buffer (≈4 mg/mL proteins) and incubated with 1 nM [^{3}H]DHA in the presence of 5 nM CGP 26505. After incubation at 25 °C for 30 min, the samples were filtered on Whatman GF/B glass-fiber filters and washed with 3 × 5 mL of phosphate buffer, dried, and added to 8 mL of Ready Protein Beckman scintillation cocktail. Nonspecific binding was measured in the presence of 35 μM l-isoprenaline.

The affinity of drugs for specific binding sites was expressed as the molar concentration inhibiting the specific binding by 50% (IC_{50}). These values were calculated from the displacement curves by log probit analysis. The dissociation constant (K_i) was derived from the equation of Cheng and Prusoff.[17] The ligand affinity (K_d) of [^{3}H]DHA was 1 nM.

Pharmacological Methods. Isolated Rat Vas Deferens. Adult male Sprague–Dawley albino rats (200–250 g body weight) were employed to test the activity of the compounds on α_1 adrenoceptors. The animals were sacrificed by cervical dislocation, their abdomen was promptly opened, and vasa deferentia were dissected, freed from the surrounding tissues, and placed in a capsula petri containing Tyrode solution at 37 °C and gassed with carbogen. For the additional experiments carried out on reserpinized animals, the tissues were obtained from rats pretreated with reserpine (2.5 mg/kg of body wt, ip) 24 h before the sacrifice. The organs, 30 mm in length, were tied at the extremities and then placed in an isolated organ bath containing Tyrode solution at 37 °C gassed with carbogen. The prostatic end of the organs was tied to a muscle holder while the epididimal end was attached to an isotonic force displacement transducer (Basile Model 7006) connected to a microdynamometer (Basile Model 7050) to record the organ responses to drug administration. The preparations were submitted to a tension of 0.5 g and left to equilibrate for 1 h before starting the experiments; a dose–response curve to norepinephrine (NE) was carried out and then the activity of the compounds to be tested was evaluated. The agonistic effect of these drugs resulted from their ability to contract the vasa deferentia smooth muscles in a dose-dependent manner. All the dose-effect curves were obtained with the method of cumulative doses, in accordance with the method described by Van Rossum.[18]

Guinea Pig Ileum. Portions of ileum were taken from male Durkin-Hartley guinea pigs (250–300 g) deprived of food intake for 24 h before the experiments. The animals were killed by cervical dislocation, their abdomens were opened, and at least

two portions (30 mm in length) of ileum were dissected about 10 cm from the ileocecal valve and freed from the adherent connective tissues. Then the specimens were placed in a 10-mL isolated organ bath, tied by the opposite extremities to a holder and an isotonic transducer. The holder had two laminar platinum electrodes placed at a distance of 7 mm which were connected to a digit stimulator (Biomedica Mangoni Model BM-ST3). The stimulation parameters were single rectangular pulses of 0.1-Hz frequency, 0.3-ms pulse width, and 12-V supramaximal voltage. The perfusion fluid was Tyrode, maintained at 37 °C and aerated with carbogen. The organs were submitted to a load of 0.5 g, left at rest for 1 h, and then electrically stimulated to obtain acetylcholine release and consequently smooth muscle contraction. α_2 agonists were evaluated for their ability to inhibit acetylcholine release and ileum contraction under selective stimulation of cholinergic nervous fibres. Cumulative dose-effect curves were obtained. α_2 antagonists were expected to increase acetylcholine release and muscle contraction.

Guinea Pig Atria. The atria were taken from the same guinea pigs as the previous preparations and employed to test the activity of the compounds on β_1 adrenoceptors. After sacrifice, the heart was removed and immersed in Tyrode solution at 37 °C aerated with pure O_2. Both the atria were separated from the ventriculi and a strip was obtained. The ends of the specimen were tied and suspended in a muscle chamber. The upper part of the organ was attached to an isometric transducer (Basile Model 7005) connected to a microdynamometer and was left to stabilize for 30 min. β_1 agonists increased the contractility of spontaneously beating atria and they were tested after a single dose. A dose–effect curve for ISO was plotted. The activity of the β_1 antagonists was assayed as their ability to reduce the inotropic response to a submaximal concentration of ISO after an incubation period of 30 min.

Guinea Pig Trachea. Tracheae were obtained from the same animals described above; the organs were transversely cut to obtain zigzag strips in accordance with the method of Emmerson and Mackay.[19] The strips were placed in an isolated organ bath as described for previous preparations, filled with Krebs solution at 37 °C, and gassed with carbogen. The organs, held at a tension of 0.5 g, were tied to an isotonic transducer and the last in turn to a microdynamometer to record the response of the smooth musculature. After a 1 h stabilizing time interval, the organ was contracted with carbachol (5.5×10^{-6} M) to obtain a marked muscular tone that made the relaxations more evident. β_2 agonists were able to relax tracheal muscular tone, and dose–response curves to ISO were obtained. The antagonism of the compounds under test on β_2 tracheal adrenoceptors was measured by their ability to inhibit the relaxing effect of a submaximal dose of ISO.

Agonist activity was expressed in terms of pD_2 values (-log ED_{50}) and intrinsic activity (the ratio between the maximal response of a test compound and that of the reference agonist, which was NE for α receptors and ISO for β receptors). The potency of the antagonists was expressed as -log IC_{50}, that is the negative logarithm of the concentration that reduced the agonist response by 50%. Reserpine was used as the free base, whereas the following drugs were used as salts: **1** (*l*-norepinephrine) as the bitartrate, **2** (*l*-isoprenaline), as the hydrochloride, the 1-AMDICs **11–14** as the hydrobromides, and carbachol as the chloride.

Molecular Graphics. The molecular models shown in Figures 2 and 7 were drawn by using the Insight II program.[10] The molecular volumes were the solvent-accessible ones, calculated by Connolly's method;[20] in Figures 2 and 6 the volumes of models **A**, **B**, and $\mathbf{B_1}$ were calculated for R = H and the volumes of model **C** and of superimposition C_1 were calculated for R = *i*-Pr.

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Supplementary Material Available: Figure 7, showing 2D NOESY spectrum of compound **20**, and Figure 8, showing selected traces corresponding to the protons H(1), H(3a), NH_2, H(5), and H(8) of compound **20** (2 pages). Ordering information is given on any current masthead page.

References

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