Synthesis and Pharmacological Investigation of the Enantiomers of Muscarone and Allomuscarone

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A strategy based on the use of (*R*)- and (*S*)-lactic ester as starting materials allowed the synthesis of the two enantiomers of muscarone [(−)-1 and (+)-1] and allomuscarone [(−)-5 and (+)-5] in >98% enantiomeric excess. The compounds were examined for their ability to bind to membranes from cerebral cortex (M_1), heart (M_2), and salivary glands (M_3) and to recognize affinity agonist states of the muscarinic receptors. The two pairs of enantiomers were also tested in five functional assays, and their muscarinic potency was determined. In both binding and functional tests, (−)-1 (2*S*,5*S*) and (−)-5 (2*R*,5*S*) were the eutomers of muscarone and allomuscarone, respectively. The eudismic ratio of muscarone, evaluated in the functional tests, spanned a range of 280–440. These values are substantially different from ones (2.4–10.1) reported in the literature. From a stereochemical point of view, muscarone behaves as muscarine and all other major muscarinic agonists; as a consequence, the hypotheses advanced to account for the anomalies of muscarone no longer have reason to exist.

A common feature among the major muscarinic agonists is the high stereoselectivity of action and a spatial arrangement around the common chiral center(s) equal to that of natural muscarine.[1-4] For many years, the sole exception was represented by muscarone (1) (Figure 1), the most potent agonist so far tested, which was regarded as an enigma[5] since it was found to possess a reversed enantioselectivity and a very low eudismic ratio (ER). This problem attracted the attention of several workers, and some explanations were proposed;[5-8] hypotheses of which are reported in the major reviews concerning cholinergic ligands.[2-4] In 1971, Bollinger and Eugster,[9] in an extensive investigation on the stereochemistry of muscarines and muscarones, reported a revision of previously assigned structures demonstrating that the eutomer of muscarone was the levorotatory isomer [(−)-1] with a configuration (2*S*,5*S*) at the chiral centers. In this regard, muscarone was not anymore an exception since it shared the same stereochemistry of natural muscarine [(+)-2 (2*S*,4*R*,5*S*)] and, despite different notations, the same stereochemical arrangement as other muscarinic ligands, i.e. (+)-3 and (+)-4 (Figure 2).

The second parameter which differentiated muscarone from the other partners was the low ER value, which, in a variety of functional tests, lay in the range of 2.4–10.1.[6,10] Usually, the eutomer is 2 orders of magnitude more active than the distomer.[3,11,12]

Since the pharmacological profile of muscarone has also been rationalized on the basis of its conformation in the solid state,[5] we have recently carried out conformational studies on a series of pentacyclic muscarinic agonists, e.g. 1–4, by means of X-ray analyses and molecular mechanics calculations.[13] The results of such an investigation put in evidence a close similarity of the conformational profile of 1 with that of its analogues 2–4. For this reason we decided to prepare the pairs of enantiomers of muscarone (1) and allomuscarone (5) in a well-established enantiomeric excess and to reinvestigate their pharmacology. This paper deals with the synthesis of (−)-1, (+)-1, (−)-5, and (+)-5 in >98% enantiomeric excess (ee) and the pharmacological investigation of their activities.

Chemistry

The synthesis of the enantiomeric pairs of 1 and 5 was achieved by employing the commercially available natural and "unnatural" lactic esters which were transformed into (2*S*)- and (2*R*)-2-(benzyloxy)propanal [(−)-6 and (+)-6]

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(1) Wang, P.-C.; Joulliê, M. M. Muscarine Alkaloids. *The Alkaloids*; Brossi, A., Ed.; Academic Press: New York, 1984; Vol. XXIII, pp 327–380.
(2) Casy, A. F. Stereochemical Aspects of Parasympathomimetics and their Antagonists: Recent Developments. *Prog. Med. Chem.* **1975**, *11*, 1–65.
(3) Dahlbom, R. Stereoselectivity of Cholinergic and Anticholinergic Agents. *Stereochemistry and Biological Activity of Drugs*; Ariëns, E. J., Ed.; Blackwell Scientific: Oxford, 1983; pp 127–142.
(4) Triggle, D. J.; Triggle, C. R. Ligands Active at Cholinergic Receptors. *Chemical Pharmacology of the Synapse*; Academic Press: New York, 1976; pp 315–324.
(5) Pauling, P.; Petcher, T. J. Muscarone: An Enigma Resolved? *Nature New Biol.* **1972**, *236*, 112–113.
(6) Waser, P. G. Chemistry and Pharmacology of Muscarine, Muscarone, and Some Related Compounds. *Pharmacol. Rev.* **1961**, *13*, 465–513.
(7) Belleau, B.; Puranen, J. Stereochemistry of the Interaction of Enantiomeric 1,3-Dioxolane Analogs of Muscarone with Cholinergic Receptors. *J. Med. Chem.* **1963**, *6*, 325–328.
(8) Beckett, A. H. Stereospecificity in the Reactions of Cholinesterase and the Cholinergic Receptor. *Ann. N.Y. Acad. Sci.* **1967**, *144*, 675–688.
(9) Bollinger, H.; Eugster, C. H. Muscarine and Related Compounds. 37. Configuration Relations in Muscarines. Chirality of *epi*-, *allo*-, and *epiallo*-muscarine, muscarone, and *allo*-muscarone. Biogenesis of Muscarine. *Helv. Chim. Acta* **1971**, *54*, 2704–2730.
(10) Gyermek, L.; Unna, K. R. Spectrum of Action of Muscarone and its Derivatives. *J. Pharm. Exp. Ther.* **1960**, *128*, 30–36.
(11) De Micheli, C.; De Amici, M.; Pratesi, P.; Grana, E.; Santagostino Barbone, M. G. Relative Potencies of the Stereoisomers of Bethanechol on Muscarinic Receptors. *Farmaco Ed. Sci.* **1983**, *38*, 514–520.
(12) Teodori, E.; Gualtieri, F.; Angeli, P.; Brasili, L.; Giannella, M.; Pigini, M. Resolution, Absolute Configuration, and Cholinergic Enantioselectivity of (+)- and (−)-cis-2-Methyl-5-[(dimethylamino)methyl]-1,3-oxathiolane Methiodide. *J. Med. Chem.* **1986**, *29*, 1610–1615.
(13) Carroll, P. J.; De Amici, M.; De Micheli, C.; Toma, L. Conformational Studies of Muscarone Analogs by X-Ray Analysis and Molecular Mechanics Calculations. *J. Med. Chem.* **1992**, *35*, 305–309.

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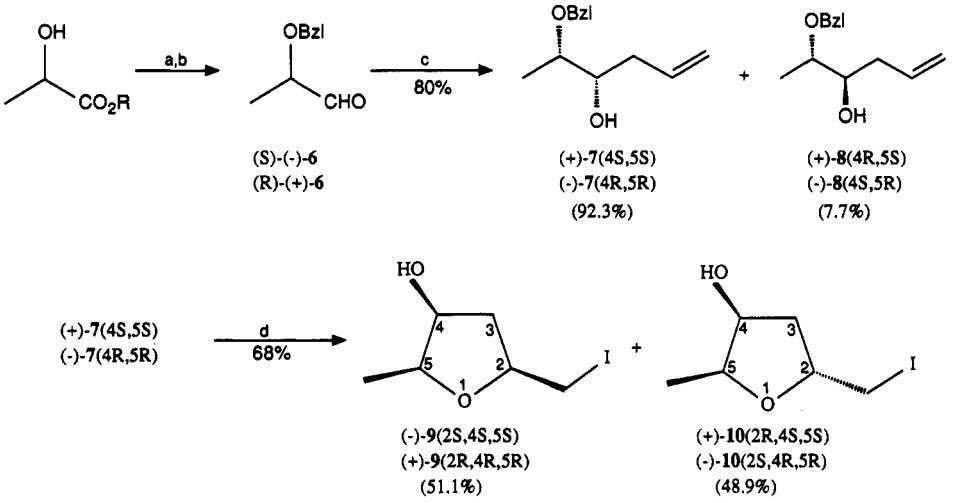
Scheme I



a: $C_6H_5CH_2Br/Ag_2O$; b: DIBAH; c: $CH_2=CHCH_2SiMe_3/SnCl_4$; d: I_2/MeCN.

Table I. Binding Affinities (μM) and Hill Coefficients (nH) of the Enantiomers of 1, 2, and 5[a]

		M_1		M_2		M_3	
compd	stereochem	K_i	nH	K_i	nH	K_i	nH
(-)-1	2S,5S	1.6	0.79[b]	0.09	0.58[b]	0.69	0.78[b]
(+)-1	2R,5R	18.6	1.0	5.64	0.60[b]	9.55	1.0
(+)-2	2S,4R,5S	15.0	0.76[b]	0.49	0.54[b]	21.60	0.82[b]
(-)-2	2R,4S,5R	220.0	1.0	104.4	0.82[b]	252.0	1.0
(-)-5	2R,5S	2.9	0.74[b]	0.14	0.58[b]	2.94	0.92[b]
(+)-5	2S,5R	42.0	0.89[b]	4.00	0.63[b]	21.18	0.90[b]

[a] Receptor source; M_1, cerebral cortex; M_2, heart; M_3, submandibular glands. K_i's are the geometrical means of three to four experiments, each performed in triplicate. [b] Significantly less than unity, $p > 0.01$.

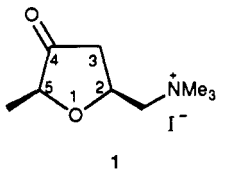


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Figure 1.

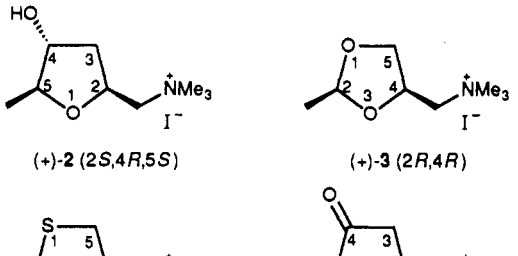


Figure 2.

(Scheme I), according to literature methods.[14-16] $SnCl_4$-mediated addition of allyltrimethylsilane to *O*-benzyllactic aldehydes (-)-6 and (+)-6 afforded a 92.3:7.7 mixture of 7 and 8 (Scheme I). The two couples of stereoisomers 7/8 were easily separated by flash chromatography, and their ratio was evaluated by capillary GLC analysis. The *threo* diastereomers (+)-7 and (-)-7 were cyclized at -15 °C with iodine in acetonitrile to produce comparable amounts of tetrahydrofurans 9 and 10. These isomers were separated by column chromatography and characterized by ^1H NMR and specific rotation.[17] HPLC analysis of the (*R*)-(+)-MTPA esters[18] of 9 and 10 showed an enantiomeric excess higher than 98%. Intermediates 9 and 10 were oxidized by pyridinium chlorochromate (PCC) to yield the two enantiomeric pairs of iodo ketones 11 and 14 (Scheme II) in good yield. The heterocyclic nucleus of these iodo ketones is particularly labile in alkaline conditions. As a consequence, compounds 11 and 14 were preliminarily transformed into dimethyl ketals 12 and 15 by a treatment with methanol in the presence of trimethyl orthoformate and *p*-toluenesulfonic acid. The subsequent nucleophilic substitution of the iodo group with dimethylamine proceeded smoothly and yielded directly tertiary amines 13 and 16 due to the regeneration of the carbonyl group during the workup procedure. A final treatment of the tertiary bases with methyl iodide produced (-)-1, (+)-1 and (+)-5, (-)-5 in quantitative yield (Scheme II). The first step of a conceivable racemization process should imply a loss of chirality at C-5; compounds

(14) Mislow, K.; O'Brien, R. E.; Schaefer, H. Stereochemistry of (+)-(S)-2-Propanol-1-d_3. Partial Asymmetric Reduction of 4',1''-Dimethyl-1,2,3,4-dibenzcyclohepta-1,3-diene-6-one. *J. Am. Chem. Soc.* **1962**, *84*, 1940–1944.

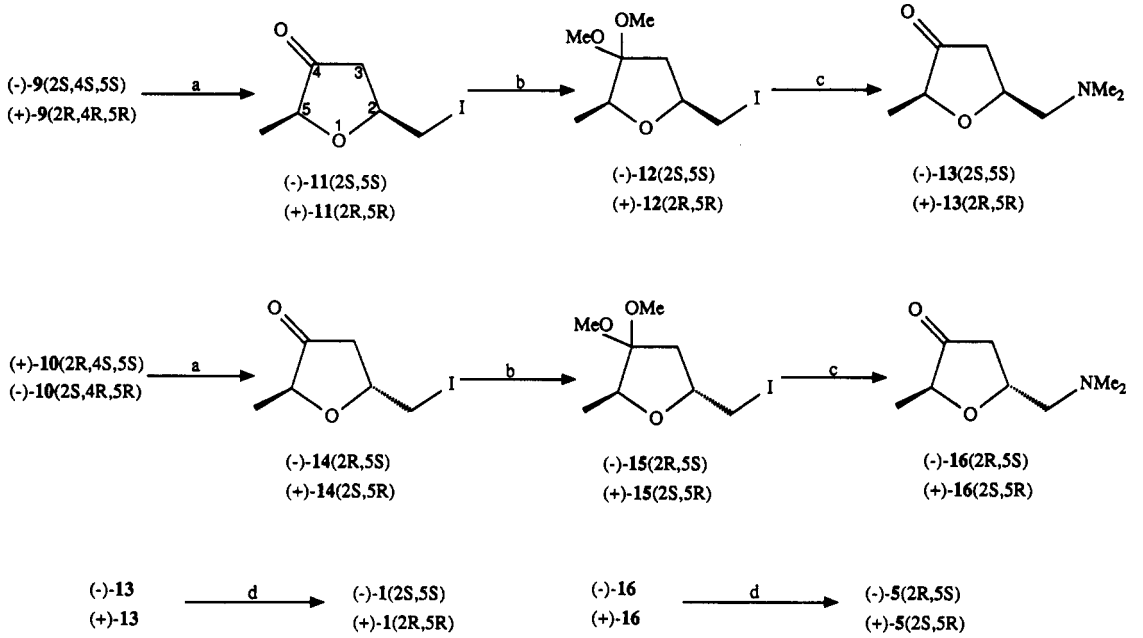
(15) Massad, S.; Hawkin, L. D.; Baker, D. C. A Series of (2S)-2-O-Protected-2-hydroxypropanals (L-Lactaldehydes) Suitable for Use as Optically Active Intermediates. *J. Org. Chem.* **1983**, *48*, 5180–5186.

(16) Ireland, R. E.; Thaisrivongs, S.; Dussault, P. H. An Approach to the Total Synthesis of Aplysiatoxin. *J. Am. Chem. Soc.* **1988**, *110*, 5768–5779.

(17) De Amici, M.; De Micheli, C.; Molteni, G.; Pitrè, D.; Carrea, G.; Riva, S.; Spezia, S.; Zetta, L. Chemoenzymatic Synthesis of the Eight Stereoisomeric Muscarines. *J. Org. Chem.* **1991**, *56*, 67–72.

(18) Dale, J. A.; Dull, D. L.; Mosher, H. S. α-Methoxy-α-trifluoromethylphenylacetic Acid, a Versatile Reagent for the Determination of Enantiomeric Composition of Alcohols and Amines. *J. Org. Chem.* **1969**, *34*, 2543–2549.

Scheme II



a: PCC/CH_2Cl_2; b: $(MeO)_3CH/MeOH/H^+$; c: $Me_2NH/MeOH$; d: MeI/Et_2O.

Table II. "In Vitro" Functional Studies of Enantiomers of 1, 2, and 5[a]

	guinea pig						rat			
compd	atria	ER[b]	trachea	ER[b]	ileum	ER[b]	jejunum	ER[b]	bladder	ER[b]
(-)-1	8.92 ± 0.05		8.95 ± 0.14		9.10 ± 0.07		8.13 ± 0.03		7.90 ± 0.07	
		417		436		380		398		282
(+)-1	6.30 ± 0.05		6.31 ± 0.05		6.52 ± 0.04		5.53 ± 0.04		5.45 ± 0.05	
(+)-2	7.69 ± 0.05				8.13 ± 0.02					
		331				324				
(-)-2	5.17 ± 0.09				5.62 ± 0.10					
(-)-5	8.29 ± 0.04				8.46 ± 0.09		7.69 ± 0.02		6.98 ± 0.04	
		45				43		32		19
(+)-5	6.64 ± 0.05				6.83 ± 0.03		6.18 ± 0.05		5.70 ± 0.04	

[a] Potencies are expressed as $-\log EC_{50}$; given are the means ± SEM. The number of experiments varied from six to nine. All the compounds possess intrinsic activity (α) equal to 1. [b] Eudismic ratio.

Table III. Affinities Constants (nM) of Compound (-)-1 and (+)-1 for the Component Agonist States of the Muscarinic Receptor Subtypes[a]

receptor component	(-)-1			(+)-1		
	M_1	M_2	M_3	M_1	M_2	M_3
K_{SH}		5.5			703	
		(2.3–12.9)			(390–1210)	
$\int_{SH}$[b]		28.4 ± 2.6			39 ± 1.3	
K_H	64.0	68.6	147		12240	
	(51–80)	(36.9–127.8)	(81–256)		(10 820–18 320)	
$\int_H$[b]	12.0 ± 1.2	49.5 ± 1.8	36.0 ± 3.1		45.6 ± 2.1	
K_L	2144	2101	1333	18620	188300	9530
	(1822–2515)	(1103–4004)	(1031–1712)	(15 130–22 900)	(100 380–370 650)	(6920–13 180)
$\int_L$[b]	86.0 ± 3.1	21.9 ± 1.3	62.0 ± 4.3		16.4 ± 1.2	

[a] The data represent the geometrical means and 95% confidence limits (in brackets) of three to four experiments, each performed in triplicate. [b] Percentage of total receptors (means ± SEM).

11 and 13 should generate, at least in part, allo derivatives 14 and 16, and vice versa. Careful capillary gas chromatographic analyses carried out on (-)-11, (-)-13, (-)-14, and (-)-16 did not give evidence of the existence of such an isomerization process. As a consequence, we can safely attribute to the final derivatives (-)-1, (+)-1, (-)-5, and (+)-5 an enantiomeric excess higher than 98%.

Results and Discussion

The muscarinic activity of the two pairs of enantiomers 1 and 5 has been evaluated both in binding studies (Table I) and isolated tissue preparations (Table II). Binding affinity (K_i) of (-)-1, (+)-1, (-)-5, and (+)-5 for *m*-AcChR subtypes was evaluated on cerebral cortex (M_1), heart (M_2), and submandibular glands (M_3). These data are compared with the corresponding values for the enantiomers of muscarine (2), the classical reference compound. Since the Hill coefficients (nH) of (-)-1, (+)-1, (-)-5, and (+)-5 were significantly less than unity, we performed one-, two-, or three-component analysis of the data, and the results are reported in Tables III and IV. Whereas (-)-1 recognized different affinity states in all three muscarinic receptor

Table IV. Affinities Constants (nM) of Compound (−)-5 and (+)-5 for the Component Agonist States of the Muscarinic Receptor Subtypes[a]

receptor component	(−)-5			(+)-5		
	M_1	M_2	M_3	M_1	M_2	M_3
K_{SH}		6.5 (4.9–8.1)				
$\int_{SH}$[b]		23.3 ± 2.1				
K_H	96 (79–118)	113 (98–132)			360 (273–488)	
$\int_H$[b]	15.1 ± 2.0	47.2 ± 4.1			37.2 ± 3.2	
K_L	4143 (3210–5733)	2095 (1471–3015)	2767 (1953–3851)	40 510 (29 610–53 620)	10 940 (8230–13 570)	21 760 (18 920–24 630)
$\int_L$[b]	83.0 ± 3.2	27.9 ± 3.1			58.1 ± 4.1	

[a] The data represent the geometrical means and 95% confidence limits (in brackets) of three to four experiments, each performed in triplicate. [b] Percentage of total receptors (means ± SEM).

subtypes (M_1, M_2, M_3), (+)-1 showed multiple components only in the M_2 subtype (Table III). Similar results were obtained with the two enantiomers of allomuscarone (5). The same comparison has also been made on the potency of the compounds, in the different muscarinic preparations: guinea pig atria, trachea, and ileum and rat jejunum and bladder (Table II).

Both sets of data confirm[9] that (−)-1, whose stereochemistry (2*S*,5*S*) is the same as the active form of muscarine ((+)-2; 2*S*,4*R*,5*S*), is the eutomer. On the contrary, the eudismic ratio values (282–436) are quite different from those reported previously (2.4–10.1),[6,10] making it clear that the degree of stereoselectivity of muscarone is comparable to that of muscarine. The reason for such a difference can safely be attributed to the optical purity of the samples used by us and by the other groups.[6,10] As shown in the Chemistry section, the two pairs of enantiomers of 1 and 5 prepared by us possess ee value >98%. Further evidence on the optical purity of our compounds derives from the values of specific rotation ($[\alpha]^{20}_D$ −19.16° and +19.72° (MeOH)) which are similar to that reported by Bollinger and Eugster for (+)-1 ($[\alpha]_D$ +18.7° (H_2O)).[9] On the contrary, (−)-muscarone chloride prepared by us possesses a specific rotation ($[\alpha]^{20}_D$ −23.54°) substantially different from the values ($[\alpha]_D$ +11.2° and −11.5°)[9] of the samples used in the biological tests.[10]

In summary, muscarone (1) does not constitute an exception anymore; it behaves as a potent muscarinic agonist with the same stereochemical requirements of muscarine (2). As a consequence, the rationalizations[2-4] set forth to explain its pharmacological anomalies are no longer valid. Both ligands recognize common binding sites, with the carbonyl moiety of 1 interacting with the same receptor subsite as the hydroxy group of muscarine (2). The nature of the interaction between the functionality in position 4 of muscarine-like compounds (Figure 2) and the complementary receptor subsite ("muscarinic subsite"[19]) can affect the potency and/or the organ selectivity more than the stereochemistry. A further inspection of the data reported in Tables I and II confirms that the substituents at C-2 and C-5 of the heterocyclic ring give the best fitting of the receptor subsites when they are in a cis relationship and possess the appropriate configuration of the stereogenic centers. On the other hand, both the enantiomers of allomuscarone (5), characterized by a trans relationship among the substituents, possess only one chiral center with the appropriate stereochemical requirements. As a consequence, their potency as well as their eudismic ratio are significantly reduced when compared to muscarone. In this context, the stereogenic center C-5 seems to be more deeply involved than C-2.

(19) Gualtieri, F.; Angeli, P.; Brasili, L.; Giannella, M.; Picchio, M. T.; Romanelli, M. N.; Teodori, E. Enantioselectivity of Chiral Cholinergic Agonists and Antagonists Carrying a 1,3-Oxathiolane Nucleus. *Recent Advances in Receptor Chemistry*; Melchiorre, C., Giannella, M., Eds.; Elsevier: Amsterdam, 1988; pp 235–250.

Experimental Section

Materials and Methods. (*R*)-(+)-Methyl lactate and (*S*)-(−)-ethyl lactate were obtained from commercial suppliers and were used as such. (2*R*)- and (2*S*)-2-(benzyloxy)propanal were prepared according to literature methods;[14-16] their specific rotations ($[\alpha]^{20}_D$ −63.3° (*c* 0.936, $CHCl_3$) and $[\alpha]^{20}_D$ +64.1° (*c* 1.249, $CHCl_3$)) agreed with the value previously reported for (2*R*)-2-(benzyloxy)propanal ($[\alpha]_D$ +64.6° (*c* 3.6, $CHCl_3$)).[16] ^1H NMR spectra were recorded with a Bruker AC-E 300 (300 MHz) spectrometer in $CDCl_3$ or D_2O solution; chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz. Rotatory power determinations were carried out with a Perkin-Elmer 241 polarimeter, coupled with a Haake N-3B thermostat. Capillary GLC analyses were conducted on a gas chromatograph equipped with a Supelcowax 10 fused silica gel column (15 m, 0.25 μm) under the following conditions: 40 °C (3 min) to 110 °C (5 min), heating rate 10 °C/min, to 225 °C, heating rate 2.5 °C/min. N_2 was used as the carrier gas at 0.4 atm. HPLC analyses were performed on a chromatograph equipped with a UV detector (λ = 254 nm) and a Whatman Partisil 10 column (250 mm length, 4.6 mm i.d.); a mixture of *n*-hexane/ethyl acetate 9:1 was used as the eluent at a flow rate of 1.0 mL/min. Retention times (t_R) are expressed in minutes. TLC were carried out on commercial silica gel GF_{254} plates. Liquids were characterized by the oven temperature for Kugelrohr distillations. (*R*)-(+)-MTPA esters were prepared according to the procedure described previously.[18]

General Procedure for the Addition of Allyltrimethylsilane to (*R*)-(+)-6 and (*S*)-(−)-6. A solution of stannic chloride (11.2 mL, 0.1 mol) in dry dichloromethane (400 mL) was cooled to −78 °C, under a stream of nitrogen. To the solution was added dropwise a solution of the aldehyde (6) (0.096 mol) in dry dichloromethane (50 mL). The solution was stirred for 10 min, and then allyltrimethylsilane (16.9 mL) was added in one portion. After stirring at −78 °C for 45 min, the reaction mixture was quenched by a dropwise addition of water (50 mL). The reaction mixture was allowed to warm to room temperature and the organic phase separated. After the usual workup the residue was column chromatographed on silica gel (eluent 15% ethyl acetate/cyclohexane) to produce pure stereoisomers 7 and 8 in 80% yield. The ratio 7/8 (92.3:7.7) was evaluated by capillary GLC under the conditions reported in Materials and Methods.

(+)-7: t_R 25.30; R_f (15% AcOEt/cyclohexane) 0.378; $[\alpha]^{20}_D$ +53.92° (*c* 0.510, $CHCl_3$).

(−)-7: $[\alpha]^{20}_D$ −53.87° (*c* 0.978, $CHCl_3$) [lit.[16] $[\alpha]_D$ −49.0° (*c* 1.578, $CHCl_3$)].

(+)-8: t_R 26.46; R_f (15% AcOEt/cyclohexane) 0.336; $[\alpha]^{20}_D$ +37.40° (*c* 1.572, $CHCl_3$).

(−)-8: $[\alpha]^{20}_D$ −37.66° (*c* 1.056, $CHCl_3$).

General Procedure for Iodocyclization of (+)-7 and (−)-7. To a cold (−15 °C) stirred solution of 7 (45 mmol) in 70 mL of anhydrous acetonitrile, under nitrogen, was added dropwise a solution of iodine (15.3 g, 0.06 mol) in dry acetonitrile (200 mL). The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was treated at room

temperature with a saturated $Na_2S_2O_3$ solution. Acetonitrile was evaporated under vacuum and the aqueous layer was extracted with dichloromethane (3 × 50 mL). The organic extracts were washed twice with an aqueous saturated solution of $NaHCO_3$ and dried (Na_2SO_4). After evaporation of the solvent, the residue was flash chromatographed on silica gel (eluent: cyclohexane/ethyl acetate 7:3) to give pure **9** and **10** in 68% yield. The **9**/**10** ratio of 51.1:48.9 was evaluated by GLC analysis of the reaction mixture under the conditions reported in Materials and Methods.

(+)-**9**: colorless prisms; mp 62–63 °C; R_f 0.21 (cyclohexane/ethyl acetate 4:1); t_R 28.42; $[\alpha]^{20}_D$ +0.347° (*c* 1.142, $CHCl_3$).

(−)-**9**: $[\alpha]^{20}_D$ −0.346° (*c* 0.997, $CHCl_3$).

(+)-**10**: colorless oil; bp 100 °C (0.5 mmHg); R_f 0.14 (cyclohexane/ethyl acetate 4:1); t_R 30.67; $[\alpha]^{20}_D$ +39.86° (*c* 1.088, $CHCl_3$).

(−)-**10**: $[\alpha]^{20}_D$ −40.74° (*c* 0.763, $CHCl_3$).

(2*S*,5*S*)-5-Methyl-2-(iodomethyl)-4(2*H*)-dihydrofuranones [(−)-11]. A suspension of (−)-**9** (2.42 g, 0.01 mol), PCC (10.8 g, 0.05 mol), and sodium acetate (3.3 g, 0.04 mol) in methylene chloride (50 mL) was stirred at room temperature until disappearance of the starting material. Celite was added followed by ethyl ether (50 mL), and the resulting slurry was filtered under vacuum through a short silica gel pad and washed with ether. The filtrate was treated with a saturated solution of copper sulfate, and the organic phase was dried and evaporated. The residue was Kugelrohr distilled at 80 °C (0.5 mmHg) as a colorless liquid: yield 1.92 g (80%). The ^1H NMR spectrum of **11** was previously reported.[17]

(−)-**11**: bp 80 °C (0.5 mmHg); R_f 0.533 (cyclohexane/ethyl acetate 7:3); $[\alpha]^{20}_D$ −34.75° (*c* 1.48, CH_2Cl_2).

The same procedure applied to (+)-**9**, (+)-**10**, and (−)-**10** gave (+)-**11**, (−)-**14**, and (+)-**14**, respectively, in 76–78% yield.

(+)-**11** (2*R*,5*R*): $[\alpha]^{20}_D$ +34.69° (*c* 1.40, CH_2Cl_2).

(−)-**14** (2*R*,5*S*): bp 80 °C (0.5 mmHg); R_f 0.566 (cyclohexane/ethyl acetate 7:3); $[\alpha]^{20}_D$ −22.62° (*c* 1.39, CH_2Cl_2).

(+)-**14** (2*S*,5*R*): $[\alpha]^{20}_D$ +22.06° (*c* 1.02, CH_2Cl_2).

General Procedure for the Transformation of Iodo Ketones (+)-11, (−)-11, (−)-14, and (+)-14 into Muscarones (−)-1 and (+)-1 and Allomuscarones (−)-5 and (+)-5.

A. In a 150-mL Erlenmeyer flask equipped with a magnetic stirrer and a refluxing condenser, a mixture of iodo ketone **11** (or **14**) (1.0 g, 4.1 mmol), trimethyl orthoformate (0.95 mL, 8.6 mmol), and *p*-toluenesulfonic acid (30 mg) in methanol (70 mL) was refluxed until disappearance of the starting material (3 h). The solution was treated with ether (150 mL), washed with a saturated $NaHCO_3$ solution (2 × 10 mL), and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was Kugelrohr distilled at 80 °C (0.5 mmHg): yield 80–85%.

(−)-**12** (2*S*,5*S*): ^1H NMR ($CDCl_3$) δ 1.21 (d, 3, Me, $J_{5,Me}$ = 6.6 Hz), 1.79 (dd, 1, H-3′, $J_{2,3'}$ = 9.9 Hz, $J_{3,3'}$ = 12.6 Hz), 2.28 (dd, 1, H-3, $J_{2,3}$ = 5.5 Hz, $J_{3,3'}$ = 12.6 Hz), 3.20 (s, 3, OMe), 3.26 (s, 3, OMe), 3.22 (dd, 1, H-6′, $J_{2,6'}$ = 5.8, $J_{6,6'}$ = 11.5 Hz), 3.27 (dd, 1, H-6, $J_{2,6}$ = 1.3 Hz, $J_{6,6'}$ = 11.5 Hz), 4.00 (m, 1, H-2), 4.11 (q, 3, H-5, $J_{5,Me}$ = 6.6 Hz); R_f 0.69 (cyclohexane/ethyl acetate 7:3); $[\alpha]^{20}_D$ −27.08° (*c* 1.06, MeOH).

(+)-**12** (2*R*,5*R*): $[\alpha]^{20}_D$ +28.77° (*c* 1.09, MeOH).

(−)-**15** (2*R*,5*S*): ^1H NMR ($CDCl_3$) δ 1.17 (d, 3, Me, $J_{5,Me}$ = 6.4 Hz), 2.05 (dd, 1, H-3′, $J_{2,3'}$ = 4.8 Hz, $J_{3,3'}$ = 13.2 Hz), 2.31 (dd, 1, H-3, $J_{2,3}$ = 7.9 Hz, $J_{3,3'}$ = 13.2 Hz), 3.22 (s, 3, OMe), 3.24 (m, 2, H-6 and H-6′), 3.27 (s, 3, OMe), 4.20 (q, 1, H-5), 4.27 (m, 1, H-2); R_f 0.72 (cyclohexane/ethyl acetate 7:3); $[\alpha]^{20}_D$ −17.57° (*c* 1.814, MeOH).

(+)-**15** (2*S*,5*R*): $[\alpha]^{20}_D$ +18.59° (*c* 1.022, MeOH).

B. A sealed metal container, filled with a solution of iodo ketone dimethyl ketal **12** (or **15**) (0.7 g, 2.45 mmol) in methanol (20 mL) and excess dimethylamine, was heated at 80 °C overnight. The container was cooled at 0 °C, and the volatiles were evaporated under vacuum. The residue was taken up with trifluoroacetic acid/water (10 mL, 1:1) and stirred at room temperature for 2 h. The solution was made basic by a portionwise addition of solid K_2CO_3 and extracted with dichloromethane (4 × 15 mL). The extracts were dried (Na_2SO_4), the solvent was evaporated, and the residue was Kugelrohr distilled at 80 °C (22 mmHg): yield 62–67%.

(−)-**13** (2*S*,5*S*): R_f 0.56 ($CHCl_3$/MeOH 7:3); $[\alpha]^{20}_D$ −79.22° (*c* 1.28, MeOH).

(+)-**13** (2*R*,5*R*): $[\alpha]^{20}_D$ +77.86° (*c* 0.94, MeOH) [lit.[9] $[\alpha]_D$ +62.3° (*c* 3.6, H_2O)].

(−)-**16** (2*R*,5*S*): R_f 0.63 ($CHCl_3$/MeOH 7:3); $[\alpha]^{20}_D$ −25.91° (*c* 0.99, MeOH) [lit.[9] $[\alpha]_D$ −44.2° (H_2O)].

(+)-**16** (2*S*,5*R*): $[\alpha]^{20}_D$ +25.12° (*c* 0.578, MeOH).

C. A solution of the tertiary amine in ether was treated with an excess of methyl iodide. The precipitate was crystallized from 2-propanol.

(−)-**1** (2*S*,5*S*): mp 176.5–177.5 °C; $[\alpha]^{20}_D$ −19.16 (*c* 1.04, MeOH).

(+)-**1** (2*R*,5*R*): $[\alpha]^{20}_D$ +19.72° (*c* 1.02, MeOH) [lit.[9] $[\alpha]_D$ +18.7 (H_2O)].

(−)-**5** (2*R*,5*S*): mp 175–176 °C; $[\alpha]^{20}_D$ −22.67° (*c* 0.79, MeOH) [lit.[9] $[\alpha]_D$ −29° (H_2O)].

(+)-**5** (2*S*,5*R*): $[\alpha]^{20}_D$ +22.96° (*c* 0.96, MeOH).

Synthesis of (−)-Muscarone Chloride. (−)-**1** (0.5 g) was passed through an Amberlite IRA-400 (Cl) ion exchange resin using distilled water as eluent. The fraction containing the product was evaporated at reduced pressure and the residue was dried at 80 °C under vacuum (0.5 mmHg): yield 0.32 g (91%). Muscarone chloride, crystallized from 2-propanol/ethyl ether as colorless needles, is highly hygroscopic and by exposure to the air turned liquid. (−)-Muscarone chloride: mp 184–185 °C dec; $[\alpha]^{20}_D$ −23.54° (*c* 1.02, H_2O) [lit.[9] $[\alpha]_D$ −11.5° (H_2O)].

Pharmacology. A. Functional Experiments. Guinea pig atria, ileum, and trachea and rat jejunum and bladder were prepared according to the protocols previously reported.[20] The potencies of the compounds, reported in Table II, were evaluated with a standard methodology.[20]

B. Binding Studies. Membranes of rat cerebral cortex, heart, and submandibular glands were prepared according to the procedure reported elsewhere.[21]

The binding curves for (−)-**1**, (+)-**1**, (−)-**5**, and (+)-**5** were analyzed by a SAS-modified computer program package giving least squares fitting to a sum of up to three superimposed binding sites. The concentration of receptors in each subgroup was expressed as a percentage of total receptors, and hence there were two independent variables for the concentration of sites, but three independent variables for binding constants. In instances in which a third binding site was not found, the computation was repeated with a reduced number of variables. The IC_{50} values were converted to K_i values by correcting for the radioligand occupancy shift according to the Cheng–Prusoff equation: $k_i = IC_{50}/[1 + C/K_D]$,[22] where C and K_D represent the concentration and the dissociation constant of the radioligand used, respectively. The K_D values for NMS used in the equation were found to be 0.52 nM in heart and 0.54 nM in submandibular glands. For PZ, the K_D was found to be 14.2 nM.

Hill coefficients (nH) were calculated by linear regression analysis and assessed for statistically significant deviation from unity by the Student's *t* test. Data from the individual experiments were analyzed independently.

The results are reported in Tables III and IV.

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Registry No. (−)-**1**, 34964-91-5; (−)-**1**·Cl^-, 16451-15-3; (+)-**1**, 140145-35-3; (+)-**2**, 24570-49-8; (−)-**2**, 79827-62-6; (−)-**5**, 93380-93-9;

(20) Grana, E.; Lucchelli, A.; Zonta, F.; Santagostino-Barbone, M. G.; D'Agostino, G. Comparative Studies of the Postjunctional Activities of Some Very Potent Muscarinic Agonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1986**, *332*, 213–218.

(21) Hammer, R.; Giraldo, E.; Schiavi, G. B.; Monferini, E.; Ladinsky, H. Binding Profile of a Novel Cardioselective Muscarine Receptor Antagonist, AF-DX 116, to Membranes of Periferal Tissues and Brain in the Rat. *Life Sci.* **1986**, *38*, 1653–1662.

(22) Cheng, Y. C.; Prusoff, W. H. Relationship between the inhibition constant K_i and the concentration of inhibition which causes fifty percent inhibition (I_{50}) of an Enzymic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(+)-5, 139892-76-5; (−)-6, 81445-44-5; (+)-6, 81445-45-6; (+)-7, 94233-72-4; (−)-7, 89104-01-8; (+)-8, 94233-71-3; (−)-8, 89104-02-9; (−)-9, 102735-38-6; (+)-9, 129170-66-7; (+)-10, 102735-37-5; (−)-10, 129170-70-3; (−)-11, 139892-68-5; (+)-11, 129170-67-8; (−)-12, 139892-71-0; (+)-12, 139892-72-1; (−)-13, 501-39-3; (+)-13, 35119-42-7; (−)-14, 139892-69-6; (+)-14, 139892-70-9; (−)-15, 139892-73-2; (+)-15, 139892-74-3; (−)-16, 6793-45-9; (+)-16, 139892-75-4.

Bioreductive Fluorescent Markers for Hypoxic Cells: A Study of 2-Nitroimidazoles with 1-Substituents Containing Fluorescent, Bridgehead-Nitrogen, Bicyclic Systems

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The oxygen-sensitive bioreductive binding of 2-nitroimidazoles labeled with fluorescent side chains has been used to stain hypoxic mammalian cells selectively. Several novel compounds were synthesized with a 1-substituent containing a fluorescent, bicyclic system having a bridgehead-nitrogen atom. Additional amine and secondary alcohol substituents were also included in the link between the fluorophor and the nitroimidazole to improve water solubility. Their ability to discriminate between hypoxic and oxic cells was compared by flow cytometric analysis. A wide range of cellular fluorescence and hypoxic–oxic differentials in fluorescence was observed when compounds with indolizine fluorophors were incubated with cells, and one such compound was considered suitable for further evaluation in vivo. Two compounds with bimane fluorophors gave very little cellular fluorescence when incubated with hypoxic cells.

Measurements of the fraction of poorly oxygenated (and therefore radio-resistant) cells in tumors[1,2] could be of considerable clinical significance as optimal radiotherapy schedules could be devised for individual patients on the basis of the oxygen status of their tumors. Those patients with significant levels of hypoxic cells could be selected for treatment with hyperbaric or normobaric oxygen to reduce the proportion of hypoxic cells, or with oxygen-mimetic radiosensitizers to increase the radiosensitivity of their hypoxic cells. Alternatively, high linear energy transfer (LET) radiation could be administered, taking advantage of the reduced dependence of radiosensitivity on oxygen levels with this sort of radiation.

One approach to the identification of hypoxic cells has been to take advantage of the inhibition by oxygen of the reductive metabolism of fluorescent nitroaromatic compounds in cells.[3-11] The nitro group quenches the fluorescence of the aromatic ring system, but on bioreduction of the nitro group in hypoxic cells the ring system becomes fluorescent.[7] Numerous nitroaromatic structures have been evaluated in model systems in vitro, but many were large planar molecules with intercalating properties.[6,7,10] Although good results have been obtained with some of these compounds in vitro, their high affinity for DNA could lead to bioavailability problems in vivo. A probe for use in vivo would have to diffuse from blood vessels through several layers of well oxygenated tumor cells to reach those cells that are hypoxic. In addition, the equilibrium between fluorescent metabolites, intercalated with DNA and in free solution, could allow the fluorescent label to diffuse from the hypoxic cells to other, better oxygenated, cells and tissues.

The hypoxia-dependent bioreductive metabolism of 2-nitroimidazoles, and resultant binding of ring fragments with the side chains to cellular constituents, has been the basis of several alternative methods proposed for determining the hypoxic fraction of tumors. Various isotope labels have been evaluated including ^{3}H, ^{14}C, ^{75}Br, ^{76}Br, ^{77}Br, and ^{19}F.[11-16] In a previous paper,[17] we examined the

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(1) Gray, L. H.; Conger, A. D.; Ebert, M.; Hornsey, S.; Scott, O. C. A. The Concentration of Oxygen Dissolved in Tissues at the Time of Irradiation as a Factor in Radiotherapy. *Br. J. Radiol.* **1953**, *26*, 638–648.
(2) Churchill-Davidson, I.; Foster, C. A.; Wiernik, G.; Collins, C. D.; Piezez, N. C. D.; Skeggs, D. B. L.; Purser, P. R. The Place of Oxygen in Radiotherapy. *Br. J. Radiol.* **1966**, *39*, 321–331.
(3) Begg, A. C.; Engelhardt, E. L.; Hodgkiss, R. J.; McNally, N. J.; Terry, N. H. A.; Wardman, P. Nitroakridin 3582: A Fluorescent Nitroacridine Stain for Identifying Hypoxic Cells. *Br. J. Radiol.* **1983**, *56*, 970–973.
(4) Olive, P. L.; Durand, R. E. Fluorescent Nitroheterocycles for Identifying Hypoxic Cells. *Cancer Res.* **1983**, *43*, 3276–3280.
(5) Olive, P. L. Cellular Metabolism of Fluorescent Nitroheterocycles. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1357–1360.
(6) Stratford, M. R. L.; Clarke, E. D.; Hodgkiss, R. J.; Middleton, R. W.; Wardman, P. Nitroaryl Compounds as Potential Fluorescent Probes for Hypoxia. II. Identification and Properties of Reductive Metabolites. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1353–1356.
(7) Wardman, P.; Clarke, E. D.; Hodgkiss, R. J.; Middleton, R. W.; Parrick, J.; Stratford, M. R. L. Nitroaryl Compounds as Potential Fluorescent Probes for Hypoxia. I. Chemical Criteria and Constraints. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1344–1351.
(8) Begg, A. C.; Hodgkiss, R. J.; McNally, N. J.; Middleton, R. W.; Stratford, M. R. L.; Terry, N. H. A. Fluorescent Markers for Hypoxic Cells: A Comparison of Two Compounds on Three Cell Lines. *Br. J. Radiol.* **1985**, *58*, 645–654.
(9) Olive, P. L.; Chaplin, D. J. Oxygen and Nitroreductase-dependent Binding of AF-2 in Spheroids and Murine Tumours. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1247–1250.
(10) Hodgkiss, R. J.; Begg, A. C.; Middleton, R. W.; Parrick, J.; Stratford, M. R. L.; Wardman, P.; Wilson, G. D. Fluorescent Markers for Hypoxic Cells. A Study of Novel Heterocyclic Compounds that Undergo Bio-Reductive Binding. *Biochem. Pharmacol.* **1991**, *41*, 533–541.
(11) Raleigh, J. A.; Franko, A. J.; Koch, C. J.; Born, J. L. Binding of Misonidazole to Hypoxic Cells in Monolayers and Spheroid Culture: Evidence that a Side-Chain Label is Bound as Effectively as a Ring Label. *Br. J. Cancer* **1985**, *51*, 229–235.

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