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Stereospecificity of myofibrillar calcium sensitivity and PDE inhibition in cardiotonic thiadiazinones

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Summary — In pyridazinone or thiadiazinone cardiotonic agents with one chiral centre, the PDE inhibitory action resides mainly in one enantiomer and the myofibrillar calcium sensitization mainly in the other. This phenomenon is observed when the chiral centre is located on the pyridazinone or thiadiazinone heterocycle, but cannot be extended to structures where the chiral centre is elsewhere on the molecule. For the first time a stereoselective synthesis of a 5-substituted 3,6-dihydro-6-methyl-2*H*-1,3,4-thiadiazine-2-one has been achieved and an absolute configuration is proposed.

stereospecificity / calcium sensitivity / phosphodiesterase / thiadiazinone / heart

Introduction

Since the discovery that the cardiotonic agent sulmazole **1** enhances myofibrillar Ca^{2+}-sensitivity [1], together with other properties, considerable attention has been directed toward discovering potent cardiotonic agents for the treatment of heart failure acting selectively by this Ca^{2+}-sensitizing mechanism.

As it has become clear that in end-stage failing hearts the inotropic effects of β1-adrenergic stimulants and phosphodiesterase (PDE) inhibitors are poor or negligible compared to those seen in a healthy heart [2–4], calcium sensitization has become an attractive alternative [5, 6]. Moreover, to be effective this mechanism does not require (or provoke) an increase in the free calcium concentration. It should therefore avoid the risk of calcium overload and potentially the associated arrhythmias. In addition, experiments measuring the heat production in papillary muscles from rat [7] and rabbit [8] have demonstrated that the contractions evoked by calcium-sensitizing agents are produced more economically than those evoked by adrenergic drugs or pure PDE inhibitors, suggesting that this mode of action might be more appropriate for the failing heart.

All of the first generation agents claimed to be calcium-sensitizers have additional properties; PDE-inhibition is usually the major effect and calcium-sensitization is of secondary importance. The first positive inotropic Ca^{2+}-sensitizing agent claimed to be completely devoid of PDE inhibitory activity or any other known inotropic mechanism is BA 41899 (5-methyl-6-phenyl-1,3,5,6-tetrahydro-3,6-methano-1,5-benzodiazocine-2,4-dione) and especially its (+)-isomer, CGP 48506 [9, 10], a hitherto unknown class of structure for this type of activity.

In a previous publication [11] we reported our attempts to discover a selective calcium-sensitizer. Although we succeeded in discovering very potent calcium sensitizers, the structural modifications performed did not completely eliminate their activity as inhibitors of the sarcoplasmic-reticulum-bound PDE. Encouraged by literature data describing different activity ratios (PDE inhibition vs calcium-sensitization) for the enantiomers of sulmazole **1** [12], pimobendan **2a** [13], meribendan **2b** [14], EMD 53 998 **3** [15–21], KF 15232 **4** [22] or ORG 20494 **5** [23], and different PDE inhibitory potencies for the enantiomers of siguazodan **6** [24], and simendan **7** [23] (fig 1), we decided to resolve some of our cardiotonic thiadiazinones [11]. Excluding resolutions of sulmazole and analogous sulfoxides, and the recent benzodiazocine derivatives [9], the most relevant literature found was that relating to enantiomers generated by introduction of a methyl group into the pyridazinone or the thiadiazinone ring. Thus we wished to check if the stereospecificity for PDE inhibition versus calcium-sensitization remains when the chiral centre is introduced elsewhere on the molecule. Therefore we chose four

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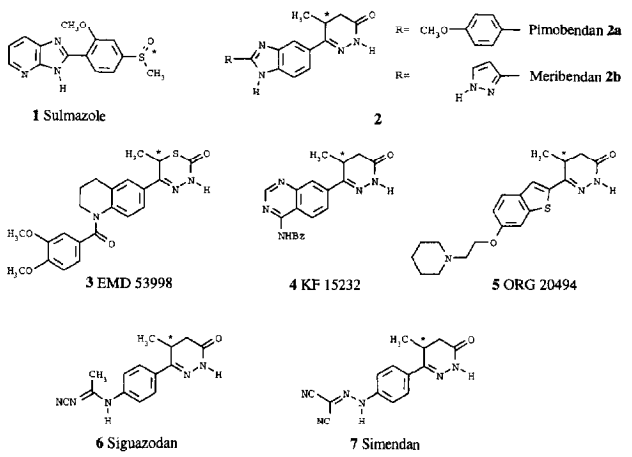


Fig 1. Cardiotonic reference compounds demonstrating stereospecificity for PDE inhibition and/or calcium-sensitization. *Chiral centre.

representative molecules, three of which bear the chiral centre on the thiadiazinone nucleus (**8–10**) and one with the chiral centre on the indole (**11**) (fig 2).

Additionally, a literature survey showed that not only has no stereoselective synthesis of 5-aryl-3,6-dihydro-6-methyl-2*H*-1,3,4-thiadiazin-2-one been described, but that no absolute configuration has been reported for such thiadiazinones, whereas many have been described for pyridazinones [22, 24, 25]. Our own attempts to obtain an interpretable X-ray pattern for the isomers of **8** failed.

Finally we succeeded in discovering a stereoselective synthesis allowing us to propose, for the first time, an absolute configuration for the enantiomers of these thiadiazinones.

Chemistry

Although a direct enantioselective synthesis of compound **8** could be proposed (scheme 1), in view of the problems encountered by others during analogous enantioselective synthesis of 6-substituted 5-methyl-4,5-dihydropyridazinones like siguazodan [24], we considered it prudent to resolve the enantiomers of **8** and **9** via a chromatographic separation of the diastereomers **12a** and **12b**, respectively, obtained by condensation with two equivalents of (–)-menthyl chloroformate (scheme 2). After deprotection, the optical purity of the isolated enantiomers was analysed by ^{1}H NMR using a chiral shift reagent as exemplified in figure 3 (see table I).

In the case of compound **11** we resolved the racemic ketone **13** by reaction with the chiral hydrazine derivative **14** [26] (scheme 3). Chromatographic

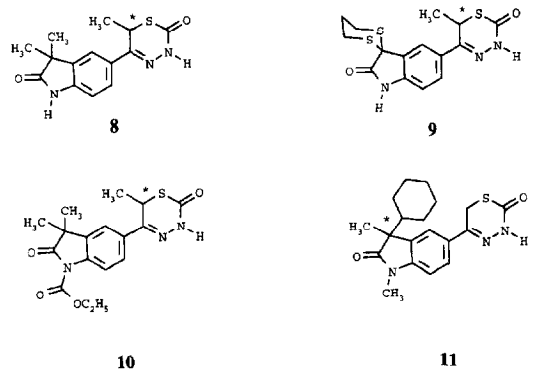
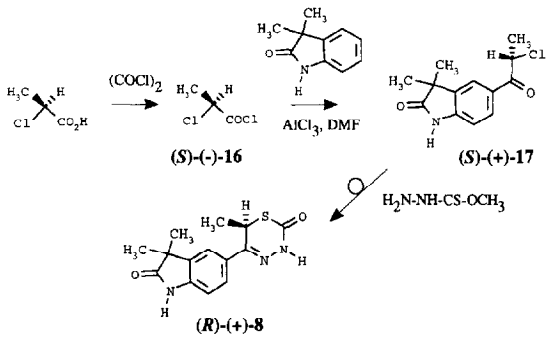
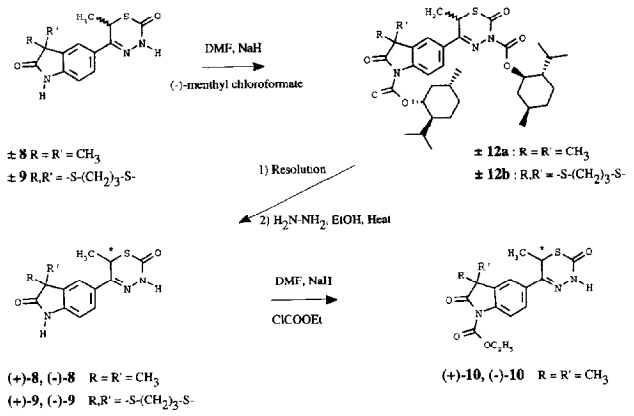


Fig 2. Cardiotonic compounds selected for isomer resolution and differentiation between PDE inhibition and calcium-sensitization.

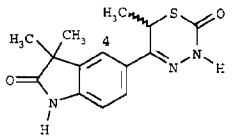


Scheme 1. Stereoselective synthesis of compound (+)-**8**.



Scheme 2. Synthetic route for the enantiomers of **8–10**.

resolution of the diastereomers **15** followed by copper sulfate deprotection [27] afforded the two enantiomeric forms of **13**. Bromination of the ketone followed by cyclization with *O*-methyl thiocarbazate [11] af-



(-) (+)

Fig 3. Part of the 200 MHz ^{1}H NMR spectrum of racemic **8** in the presence of 1.5 equiv EU(hfc)$_3$ showing the duplication of the H-4 aromatic proton at 10.9 and 11.05 ppm corresponding to the (–)- and (+)-enantiomers respectively.

forded the desired isomers with an enantiomeric excess >95% as determined by ^{1}H NMR using a chiral shift reagent as described above (table I).

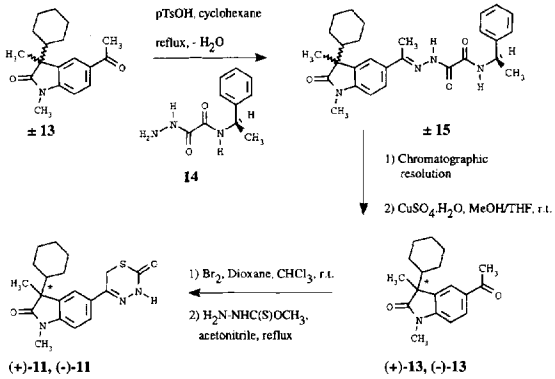
As already described, our attempts to obtain an interpretable X-ray pattern for the enantiomers of **8** failed despite apparently satisfactory crystals. Encouraged by some more recent literature information we attempted a stereoselective synthesis with the aim of determining their absolute configuration.

Since the Friedel–Crafts acylation of aromatic compounds with optically active acid chlorides had been reported to produce α-halopropiophenones in high enantiomeric purity [28], it was anticipated that this method could also serve as a starting point for our synthesis (scheme 1).

Table I. Summary of rotation and optical purity data.

Compound	$[\alpha]_D^{20}(°)$	*Solvent*	*ee*[a]
(+)-**8**	+801	DMF	98
(–)-**8**	–803	DMF	98
(+)-**9**	+673	DMF	94
(–)-**9**	–625	DMF	>80
(+)-**10**	+566	$CHCl_3$	98
(–)-**10**	–565	$CHCl_3$	98
(+)-**11**	+57.4	DMF	96
(–)-**11**	–55.2	DMF	92
(+)-**12a**	+133	$CHCl_3$	ND[b]
(–)-**12a**	–257	$CHCl_3$	ND
(+)-**12b**	+134	$CHCl_3$	ND
(–)-**12b**	–214	$CHCl_3$	ND
(+)-**13**	+6.26	$CHCl_3$	>85[c]
(–)-**13**	–7.43	$CHCl_3$	96
(+)-**15**	+38.4	$CHCl_3$	ND
(–)-**15**	–35.8	$CHCl_3$	ND
(+)-**17**	+45	$CHCl_3$	98

[a]Determined by ^{1}H NMR, see *Experimental protocols* and figure 3. [b]Not determined. [c] ^{1}H NMR did not allow more accurate estimation for this compound.



Scheme 3. Synthetic route for the preparation of the enantiomers of compound **11**.

Baine and co-workers [24] having demonstrated that Friedel–Crafts acylation with optically active 2-bromopropionyl chloride gave rise to a large ratio of racemization, we started from (*S*)-(–)-2-chloropropionyl chloride **16** obtained in one step from commercially available (*S*)-(–)-2-chloropropionic acid. An additional and important reason to use the chloro rather than the bromo derivative is that Hoffmann and Hughes [29] demonstrated that in bimolecular nucleophilic substitution of an optically active bromide by a nucleophile a significant bimolecular symmetrical exchange could occur (scheme 4) and would lead to a substantial racemization at the bromo ketone level analogous to **17**.

Substitutions of chiral halides with thioureas [29] or substitutions of tosylates with potassium ethyl xanthogenates [30] proceed with inversion of configuration and we could reasonably expect the same type of inversion in our case.

In practice, when the chloroketone (+)-**17** is heated with *O*-methyl thiocarbazate in a solvent, usually acetonitrile, compound **8** is obtained with a 30% yield and with an unexpected almost total racemization (≥99%).

It was therefore highly desirable to find a new synthetic method and we discovered eventually that heating a stoichiometric mixture of (+)-**17** and neat *O*-methyl thiocarbazate in a microwave oven led to a dramatic increase in the stereoselectivity of the reaction. Optimization of these conditions allowed us to isolate a small amount of (+)-**8** free of any detectable amount of the other enantiomer, as determined by ^{1}H NMR using a chiral shift reagent.

$$Br^- + (+)\text{ R-Br} \rightleftharpoons (-)\text{ R-Br} + Br^-$$

Scheme 4. Racemization by bromide ion.

Since inversion of configuration is expected during the cyclization step with *O*-methyl thiocarbazate we propose that the configuration of this isomer is *R* (scheme 1).

Biological results and discussion

Biological evaluation of the isomeric pairs of each compound was undertaken in experiments in vitro. Enantiomers of each pair were compared for their potency in inhibiting the sarcoplasmic reticulum-bound low K_m cAMP phosphodiesterase (SR-PDE) [31] prepared from dog left ventricule, and their influence on Ca^{2+}-dependent ATPase activity of canine cardiac myofibrils.

In table II, the inhibitory concentrations of the compounds on SR-PDE are expressed as IC_{50} values (50% inhibition) and their activity as calcium-sensitizers as ΔpCa, the shift for the concentration(s) shown for each compound of the Ca^{2+} concentration (expressed as its negative logarithm) required for half-maximal activation of the cardiac myofibrillar ATPase.

For the three pairs of compounds in which the chiral centre is on the thiadiazinone, (+)(−)-**8**, (+)(−)-**9**, (+)(−)-**10**, the (−)-isomer appears to be approximately 20-fold more potent SR-PDE inhibitor than the (+)-isomer. This stereoselectivity for PDE inhibition is in agreement with data reported in literature for pimobendan, EMD 53998, simendan, meribendan, siguazodan and KF 15232 [13, 15–20, 22–24]. It can therefore be claimed as a general rule in the diazinone series that the strongest PDE inhibition is supported by the (−)-isomer. Our results suggest that in the thiadiazinone series the (*S*)-configuration supports the highest inhibition of PDE activity, which is in agreement with the pyridazinone series where the (*R*) configuration seems to be essential for the inhibition of PDE [22, 24], (see fig 4).

Regarding the Ca-sensitizing effect, for the three pairs of compounds in which the chiral centre is on the thiadiazinone, this effect resides only in the (+)-isomer. This stereoselectivity for the Ca-sensitizing effect is in agreement with other literature data in the thiadiazinone series [15–20]. This confirms an unambiguous stereoselectivity in this class, in contrast to a less clear-cut stereoselectivity for the Ca-sensitizing effect in the pyridazinone series, for compounds such as meribendan [14] and simendan [23]. Furthermore, our results provide the first demonstration that in the thiadiazinone series, the (*R*)-configuration may be essential for the Ca-sensitizing effect.

Surprisingly this discrimination was not observed for the pairs (+)- and (−)-**11**. The stereoselectivity between these isomers is less marked and a single isomer, (−)-**11**, was found to be the more potent on both enzymatic activities.

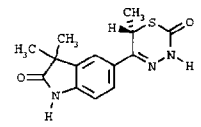
The fact that the chiral centre in **11** is more distant from the thiadiazinone nucleus might explain this different behaviour as we clearly demonstrated the essential role of this heterocycle in calcium-sensitization [11]. The low influence of a chiral centre more distant from the pyridazinone/thiadiazinone cycle is fully compatible with the topographical model of the cardiac cAMP phosphodiesterase receptor proposed by Erhardt and co-workers [32].

To optimize the difference between the (+)- and the (−)-isomers we first attempted to synthesize the analogue of compound **11** without the methyl in position 3 of the indolone (compound **18**, scheme 5). Unfortunately the cyclization at the last step induced a very high rate of racemization at indole C-3 as well as thiadiazine C-6. This was confirmed by adding one equivalent of D_2O in the reaction mixture (scheme 5).

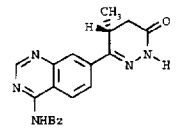
Table II. Summary of in vitro results.

Compound	*Inhibition of dog SR-PDE*	*Dog myofibrillar ATPase*	
	(IC_{50}[a], μM)	*ΔpCa_{50}[b]*	*[μM]*
(+)-**8**	4.30	0.64 ± 0.14	200
(−)-**8**	0.26	−0.06 ± 0.05	200
(+)-**9**	4.60	0.48 ± 0.02	3
(−)-**9**	0.25[c]	0.00 ± 0.04	3
(+)-**10**	1.50	0.67 ± 0.14	200
(+)-**10**	1.50	0.18 ± 0.03	30
(−)-**10**	0.05	0.03 ± 0.06	200
(+)-**11**	7.70	0.15 ± 0.06	30
(−)-**11**	1.50	0.56 ± 0.12	30

[a]IC_{50} was determined according to procedure described in the *Experimental protocols* from the mean inhibition curve obtained from three to four different SR-PDE preparations. [b]ΔpCA_{50} is the leftward shift, induced by the concentration(s) shown for each compound of the Ca^{2+} concentration (expressed as its negative logarithm) required for half-maximal activation of the cardiac myofibrillar ATPase. Values, determined as described in the *Experimental protocols* are expressed as mean ± SEM. [c]Contaminated by ca 10% mol of (+)-**6**.

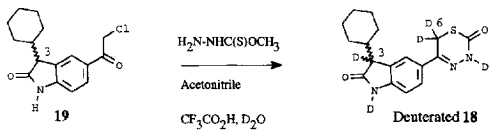


(S)-(-)-8



(R)-(-)-KF15232

Fig 4. Absolute configuration of (−)-**8** and (−)-KF15232.



Scheme 5. Racemization at indolone C-3 during formation of the thiadiazinone ring.

The indole proton at C-3, which is not exchangeable by D_2O under usual NMR conditions, was exchanged during the reaction. Substitution of the two protons at C-6 of the thiadiazinone confirmed the previously observed extensive racemization with the 6-methyl analogue under the same reaction conditions. Transient formation of enol forms at both positions is a likely explanation.

Conclusion

We have resolved the enantiomers of several cardiotonic thiadiazinones and found that the enantiomers have different mechanisms of action. For the first time a stereoselective synthesis of a 5-substituted 3,6-dihydro-6-methyl-2*H*-1,3,4-thiadiazinone has been achieved allowing us to propose an absolute configuration for the enantiomers of such compounds. This allowed us to demonstrate that in the thiadiazinone series the (*R*) configuration may be essential for the Ca-sensitizing effect. Furthermore, the stereo requirements for PDE inhibition are the same in the pyridazinone and thiadiazinone series.

Experimental protocols

Chemistry

Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were recorded in deuteriochloroform or dimethylsulfoxide-d_6 on a Bruker AC 200 MHz spectrometer. NMR titration of optical isomers were performed using tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato] europium(III) (Eu(hfc)$_3$) from Aldrich as chiral shift reagent (fig 3). The infrared spectra were of samples in potassium bromide, measured using a Shimadzu IR 408 model spectrometer.

Optical rotations were recorded on a Perkin-Elmer 141 polarimeter. Microanalytical data were provided by the Physical and Analytical Service Unit of the SmithKline Beecham Pharmaceutical Research Laboratories at Great Burgh, UK; only symbols of analysed elements are given, and data were within ±0.4% of theoretical values unless indicated.

Unless otherwise noted, a standard work-up and purification procedure was used for isolation of products. This involved filtration or extensive extraction with a solvent (washing of extract with aqueous solutions, when mentioned), drying over magnesium sulfate and evaporation under reduced pressure. Chromatographic purifications were performed using E Merck silica gel 60 (70-230 mesh) and all solvents were of Carlo Erba RPE grade; the nature and ratio of the solvent mixtures used will be reported each time.

Separation of diastereomers was performed by medium pressure preparative column chromatography (Jobin-Yvon device: 10 kg/cm^2) using TLC grade silica gel (Merck 60H, medium particle size 15 µm).

*Synthesis of (–)-menthyl chloroformate derivatives **12**. General procedure*

Sodium hydride (55% dispersion in oil, washed with hexane, 0.04 mol) was added under nitrogen to an ice-cold solution of racemic thiadiazinone **8** [33] or **9** [11] (0.02 mol) in 60 mL DMF. The suspension was stirred for an additional 0.5 h at room temperature after the end of hydrogen evolution. The mixture was cooled again to 0 °C and a solution of (–)-menthyl chloroformate (0.04 mol) in 20 mL DMF was added dropwise over 1 h. After overnight stirring, the DMF was evaporated in vacuo. Flash chromatography (heptane/ethyl acetate 4:1) afforded the diastereomeric mixture of disubstituted compounds.

Mixture **12a**: yield: 77%. IR (KBr): ν = 2980, 1775, 1680 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ = 0.7–2.3 (m, 45H, 2 x menthyl + 3 x CH_3), 4.29 (q, *J* = 7.3 Hz, 1 H, CH), 4.8–5.1 (m, 2H, 2 x OCH), 7.64 (dd, *J* = 8.6 Hz, *J'* = 1.8 Hz, 1H, Ar), 7.80 (d, *J'* = 1.8 Hz, 1H, Ar), 7.97 (d, *J* = 8.6 Hz, 1H, Ar).

Mixture **12b**: yield: 37%. IR (KBr): ν = 2990, 1775, 1738, 1680 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ = 0.7–2.3 (m, 47H, 2 x menthyl + CH_2 + 3 x CH_3), 2.7–2.9 (m, 2H, SCH_2), 3.6–3.8 (m, 2H, SCH_2), 4.6–5.0 (m, 3H, 2 x OCH + CH), 7.85–8.10 (m, 3H, Ar).

Separation of the diastereomers

Chromatography: ratio compound/silica 1:100 and heptane/ethyl acetate 10:1 as eluent. See table I for rotation values. (+)-**12a**: yield: 18%. (–)-**12a**: yield: 19%. (+)-**12b**: yield: 33%. (–)-**12b**: yield: 33%.

*Recovery of the enantiomers **8** and **9**. General procedure*

The carbamate (0.01 mol), and hydrazine (0.04 mol) in ethanol (20 mL) were stirred at 70 °C for 2 h. The mixture was concentrated in vacuo, taken up in diethyl ether/ethyl acetate 4:1 (100 mL) and washed twice with water (50 mL). Evaporation in vacuo and crystallization from diethyl ether afforded a pure compound. Optical rotations are reported in table I.

(+)-**8**: yield: 59% (from (+)-**12a**); mp: 270 °C. ^{1}H NMR ($CDCl_3$): δ = 1.45 (s, 6H, 2 x CH_3), 1.69 (d, *J* = 7.3 Hz, 3H, CH_3), 4.27 (q, *J* = 7.3 Hz, 1H, CH), 6.98 (d, *J* = 8.2 Hz, 1H, Ar), 7.52 (dd, *J* = 8.2 Hz, *J'* = 1.7 Hz, 1H, Ar), 7.69 (d, *J* = 1.7 Hz, 1H, Ar), 8.44 (s, 1H, exch D_2O, NH), 9.08 (s, 1H, exch D_2O, NH). (–)-**8**: yield: 57% (from (–)-**12a**); mp, IR and ^{1}H NMR were identical to the other isomer.

For compounds (+)-**9** and (–)-**9**, deprotection was carried out at room temperature. (+)-**9**: yield: 60% (from (+)-**12b**); mp, IR and ^{1}H NMR are identical to the racemic one [8]. (–)-**9**: yield: 46% (from (–)-**12b**); mp, IR and ^{1}H NMR are identical to the other isomer.

*Ethyl (+)- and (–)-2,3-dihydro-5-(3,6-dihydro-6-methyl-2-oxo-2H-1,3,4-thiadiazin-5-yl)-3,3-dimethyl-2-oxo-1H-indol-1-carboxylate **10***

A solution of (+)- or (–)-**8** (5 mmol) in 30 mL DMF was cooled to 0 °C. NaH (5 mmol, from a 55% dispersion in oil) was added and the mixture was stirred successively for 0.5 h at 0 °C and 0.5 h at 20 °C. After cooling to 0 °C, a solution of ethyl

chloroformate (5.5 mmol) in DMF (2 mL) was added dropwise. The mixture was stirred overnight at room temperature, ethyl acetate (250 mL) was added and the organic phase was washed with water. Chromatography: methylene chloride/ethyl acetate 4:1.

(+)-**10**: yield 48% (from (+)-**8**). mp: 170 °C. IR: ν = 3250, 1755, 1735, 1655, 1620 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.44–1.51 (m, 9H, CH_3), 1.69 (d, *J* = 7.3 Hz, 3H, CH_3), 4.30 (q, *J* = 7.3 Hz, 1H, CH), 4.50 (q, *J* = 7.1 Hz, 2H, CH_2), 7.59 (dd, *J* = 8.6 Hz, *J'* = 2.0 Hz, 1H, Ar), 7.72 (d, *J'* = 2.0 Hz, 1H, Ar), 7.99 (d, *J* = 8.6 Hz, 1H, Ar), 8.95 (s, 1H, exch D_2O, NH). (–)-**10**: yield 53% (from (–)-**8**). mp: 170 °C. IR and ^1H NMR are identical to the other isomer.

Stereoselective synthesis of (+)-8

*(S)-(–)-2-Chloropropionyl chloride **16***. A solution of 6.1 g (48 mmol) oxalyl chloride in 15 mL methylene chloride was added dropwise to a solution of 5 g (*S*)-(–)-2-chloropropionic acid in 60 mL methylene chloride plus a few drops of DMF. Stirring was maintained 2 h after the end of the addition, the solvent was concentrated in vacuo affording 5.8 g of a crude acid chloride used as such in the next step.

*(S)-(+)-5-(2-Chloro-1-oxo)propyl-1,3-dihydro-3,3-dimethyl-2H-indol-2-one **17***. Under ice cooling 8 g of DMF was added slowly to 46 g (345 mmol) aluminium chloride. The mixture was stirred 0.5 h at 60 °C and cooled to room temperature. Then, 5.8 g (36.2 mmol) 1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one were added neat, followed by 4.7 g (36.2 mmol) of crude (*S*)-(–)-2-chloropropionyl chloride **16**. The mixture was stirred 2 h at 60 °C, and then poured onto 200 g of crushed ice, acidified with 15 mL aq 10 N HCl, and extracted twice with ethyl acetate (50 mL). The cumulated organic extract was washed three times with water (100 mL) and evaporated. The residue was triturated with diethyl ether to afford 4.6 g of white crystals. Yield: 50%. mp: 145 °C.

^1H NMR ($CDCl_3$): δ = 1.45 (s, 6H, 2 x CH_3), 1.75 (d, *J* = 6.6 Hz, 3H, CH_3), 5.24 (q, *J* = 6.6 Hz, 1H, CH), 7.01 (d, *J* = 8.2 Hz, 1H, Ar), 7.91 (d, *J'* = 1.7 Hz, 1H, Ar), 7.95 (dd, *J* = 8.2 Hz, *J'* = 1.7 Hz, 1H, Ar), 8.67 (s, 1H, exch D_2O, NH).

Addition of 2.25 equiv of $Eu(hfc)_3$ to the NMR solution confirmed the presence of only one isomer.

*(R)-(+)-1,3-Dihydro-5-(3,6-dihydro-6-methyl-2-oxo-2H-1,3,4-thiadiazin-5-yl)-3,3-dimethyl-2H-indol-2-one (+)-**8***. One micromole (251 mg) of (*S*)-(+)-5-(2-chloro-1-oxo)propyl-1,3-dihydro-3,3-dimethyl-2H-indol-2-one **17** was thoroughly mixed with 1 equiv of *O*-methyl thiocarbazate [34, 35] (106 mg) and placed without solvent in a glass test tube which was introduced in a 800 W domestic microwave oven. The power was set at half scale and the irradiation maintained 30 s after melting of the mixture. The total reaction time was approximately 2.5 min and the final temperature 105 °C. After cooling the mixture was taken up in 1 mL acetonitrile, the solid was filtered off, washed with 0.5 mL acetonitrile and dried to afford 60 mg (21%) of the title compound.

$[\alpha]_D^{20}$ = + 692° (c = 1, DMF). ^1H NMR was identical to that of **8**. Only one isomer could be detected after addition of (+)-$Eu(hfc)_3$, so the enantiomeric excess could be estimated to be >96% (on chemically pure compounds this method is more accurate and allows the detection of less than 1% of the second isomer for **8** and **10**). The lower rotation compared to (+)-**8** obtained by the previous method is probably due to the lower purity of the sample which had been neither chromatographed nor recrystallized.

*(±)-5-Acetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2H-indol-2-one **13***

(±)-3-Cyclohexyl-1,3-dihydro-1,3-dimethyl-2H-indol-2-one. A 60% suspension of sodium hydride in oil (5 g, 140 mmol) was added slowly to a solution of 15 g (70 mmol) 3-cyclohexyl-1,3-dihydro-2*H*-indol-2-one [36] in 300 mL DMF, and stirring was continued for 0.5 h. Then 19.9 g (140 mmol) methyl iodide was added and stirring was maintained overnight at room temperature. Usual work-up and purification afforded, after chromatography (hexane/ethyl acetate 9:1), 7.84 g (46%) of the title compound.

^1H NMR ($CDCl_3$): δ = 0.74–1.03 (m, 2H, cyclohexyl), 1.04–1.31 (m, 4H, cyclohexyl), 1.34 (s, 3H, CH_3), 1.45–1.66 (m, 2H, cyclohexyl), 1.72–1.81 (m, 3H, cyclohexyl), 3.20 (s, 3H, NCH_3), 6.82 (d, 1H, *J* = 7.7 Hz, Ar), 7.04 (t, 1H, *J* = 7.7 Hz, Ar), 7.20 (d, 1H, *J* = 7.7 Hz, Ar), 7.26 (t, 1H, *J* = 7.7 Hz, Ar).

*(±)-5-Acetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2H-indol-2-one **13***. DMF (6.11 mL) was added slowly to 38.4 g (290 mmol) of aluminium trichloride, the mixture stirred for 0.5 h at room temperature and 7 g (29 mmol) 3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2*H*-indol-2-one were added. Acetyl chloride (2.26 g, 29 mmol) was added dropwise and the mixture stirred overnight at room temperature. Usual workup afforded 5 g (60%) of the title compound as white crystals, mp: 120 °C.

^1H NMR ($CDCl_3$): δ = 0.75–1.29 (m, 5H, cyclohexyl), 1.37 (s, 3H, CH_3), 1.45–1.88 (m, 6H, cyclohexyl), 2.60 (s, 3H, $COCH_3$), 3.24 (s, 3H, NCH_3), 6.86 (d, 1H, *J* = 8.2 Hz, Ar), 7.83 (d, 1H, *J'* = 1.5 Hz, Ar), 7.93 (dd, 1H, *J* = 8.2 Hz, *J'* = 1.5 Hz).

*(R)-Oxo[(1-phenylethyl)amino]acetic acid, [1-(3-cyclohexyl-2,3-dihydro-1,3-dimethyl-2-oxo-1H-indol-5-yl)ethylidene]-hydrazide **15***

(R)-2-Hydrazino-2-oxo-N-(1-phenylethyl)acetamide. This reagent is prepared following the method described by Suffert [26]. A mixture of 15 g (124 mmol) (*R*)-(+)-α-methylbenzenemethanamine and 38 mL (250 mmol) diethyloxalate in 150 mL ethanol is stirred for 24 h. The slight precipitate was removed by filtration, and the solvent and unreacted diethyloxalate evaporated in vacuo. This crude residue was dissolved in 270 mL ethanol and 6.75 g (135 mmol) hydrazine hydrate were added. The precipitate was filtered and recrystallized in ethanol affording 16.67 g (65%) of the title compound.

*Diastereomeric mixture of (R)-oxo[(1-phenylethyl)amino]-acetic acid, [1-(3-cyclohexyl-2,3-dihydro-1,3-dimethyl-2-oxo-1H-indol-5-yl)ethylidene]hydrazide **15***. Following the literature procedure [26] a mixture of 5.4 g (19 mmol) 5-acetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2*H*-indol-2-one, 3.93 g (19 mmol) (*R*)-oxo[(1-phenylethyl)amino]acetic acid, hydrazide, 0.2 g *p*-toluenesulfonic acid and 250 mL cyclohexane was refluxed for 7 h, the water formed being removed by a Dean-Stark device. The white suspension was chilled to give a heavy white crystalline solid, which was filtered and dried affording 5.69 g (63%) of the title compound. An additional crop could be obtained by concentration of the mother liquors and compound was also present in the gummy solid left on the walls of the reaction vessel, mp: 117 °C. Microanalysis (C, H, N) $C_{28}H_{34}N_4O_3$.

^1H NMR ($CDCl_3$): δ = 0.85–1.35 (m, 6H, cyclohexyl), 1.37 (s, 3H, CH_3), 1.60 (s, 3H, CH_3), 1.51–1.87 (m, 5H, cyclohexyl), 2.35 (s, 3H, CH_3), 3.21 (s, 3H, NCH_3), 5.11 (m, 1H, CH), 6.82

(d, 1H, J = 8.8 Hz, Ar), 7.33–7.42 (m, 5H, Ar), 7.72–7.76 (m, 2H, Ar), 7.83 (d, 1H, J = 7.9 Hz, exch D_2O, NH), 10.18 (s, 1H, exch D_2O, NH).

Chromatographic resolution of the diastereomers of (R)-oxo[(1-phenylethyl)amino]acetic acid, [1-(3-cyclohexyl-2,3-dihydro-1,3-dimethyl-2-oxo-1H-indol-5-yl)ethylidene]hydrazide ***15***. The separation was performed on the 5.69 g obtained aboved (heptane/ethyl acetate 3:1). (+)-**15**: yield: 56%; mp: 106 °C. (–)-**15**: yield: 70%; mp: 117 °C.

Recovery of the enantiomers. General procedure
One resolved diastereomer of **15** (4 mmol) and $CuSO_4 \cdot 5H_2O$ (16 mmol) were dissolved in 50 mL of a 1:1 mixture of methanol and THF and stirred for 24 h. The solvent was concentrated in vacuo, the residue taken up in water and extracted four times with 50 mL ethyl acetate. Chromatography: heptane/ethyl acetate 7:3. The resulting white oil usually crystallizes after seeding. See table I for rotation values.

(+)-**13**: yield: 91% (from (+)-**15**); mp: 91 °C. Enantiomeric evaluation by NMR showed the presence of approximately 7% of the other isomer.

(–)-**13**: yield: 61% (from (–)-**15**); mp: 95 °C.

(+)- and (–)-1,3-Dihydro-5-(3,6-dihydro-2-oxo-2H-1,3,4-thiadiazin-5-yl)-3-cyclohexyl-1,3-dimethyl-2H-indol-2-one ***11***. *General procedure*

(±)- and (–)-5-Bromoacetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2H-indol-2-one. Optically active 5-acetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2*H*-indol-2-one (+)-**13** or (–)-**13** (2 mmol) was dissolved in a mixture of 15 mL chloroform and 3 mL dioxane. The solution was heated at 60 °C under argon and a solution of 2 g of bromine in 50 mL chloroform was added dropwise over approximately 2 h. After cooling the solution was diluted with 50 mL chloroform and washed twice with 50 mL water. Evaporation of the organic phase afforded a nearly quantitative yield of the desired compound which was used without purification in the next step.

(+)- and (–)-1,3-Dihydro-5-(3,6-dihydro-2-oxo-2H-1,3,4-thiadiazin-5-yl)-3-cyclohexyl-1,3-dimethyl-2H-indol-2-one ***11***. A mixture of crude, optically active, 5-bromoacetyl-3-cyclohexyl-1,3-dihydro-1,3-dimethyl-2*H*-indol-2-one (2 mmol), *O*-methyl thiocarbazate [34, 35] (2 mmol), and trifluoroacetic acid (2 mmol) in 20 mL acetonitrile was refluxed for 3 h. Usual work-up and purification by chromatography (hexane/ethyl acetate 1:1) followed by a crystallization in diethyl or diisopropyl ether afforded the title compounds.

(+)-**11**: yield 18% (from (+)-**13**). Mp 203 °C. Microanalysis (C, H, N, S) $C_{19}H_{23}N_3O_2S$. ^1H NMR ($CDCl_3$): δ = 0.78–1.29 (m, 6H, cyclohexyl), 1.38 (s, 3H, CH_3), 1.47–1.86 (m, 5H, cyclohexyl), 3.23 (s, 3H, NCH_3), 3.99 (s, 2H, SCH_2), 6.86 (d, 1H, J = 8.2 Hz, Ar), 7.58 (dd, 1H, J = 8.2 Hz, J' = 1.6 Hz, Ar), 7.65 (d, 1H, J' = 1.6 Hz, Ar), 8.78 (s, 1H, exch D_2O, NH).

(–)-**11**: yield 16% (from (–)-**13**); mp 203 °C. ^1H NMR was identical to the other isomer.

(±)-5-Chloroacetyl-3-cyclohexyl-1,3-dihydro-2H-indol-2-one ***19***
Starting from 3-cyclohexyl-1,3-dihydro-2*H*-indol-2-one [36] and chloroacetyl chloride, the procedure used for the synthesis of compound **13** gave rise to the title compound after purification by chromatography (chloroform/ethyl acetate 95:5). Yield: 49%; mp: 165 °C.

^1H NMR (DMSO-d_6): δ = 1.02–1.60 (m, 10H, cyclohexyl), 2.04 (m, 1H, cyclohexyl), 3.45 (d, J = 3.1 Hz, 1H, CH), 5.13 (2s, 2H, CH_2), 6.92 (d, J = 8.1 Hz, 1H, Ar), 7.83 (d, J' = 1.2 Hz, 1H, Ar), 7.89 (dd, J = 8.1 Hz, J' = 1.2 Hz, 1H, Ar), 10.81 (s, 1H, exch D_3O, NH).

(±)-1,3-Dihydro-5-(3,6-dihydro-2-oxo-2H-1,3,4-thiadiazin-5-yl)-3-cyclohexyl-2H-indol-2-one ***18***
Starting from (±)-5-chloroacetyl-3-cyclohexyl-1,3-dihydro-2*H*-indol-2-one **19** and the procedure used for the synthesis of compound **11** afforded the title compound with 21% yield after usual work-up, mp: 248 °C. Microanalysis: (C, H, N, S) $C_{17}H_{19}N_3O_2S$.

^1H NMR (DMSO-d_6): δ = 1.02–1.48 m, (6H, cyclohexyl), 1.57–1.63 (m, 4H, cyclohexyl), 1.99 (m, 1H, cyclohexyl), 3.40 (d, J = 2.5 Hz, 1H, CH), 4.19 (s, 2H, SCH_2), 6.87 (d, J = 8.2 Hz, 1H, Ar), 7.65 (dd, J = 8.2 Hz, J' = 1.8 Hz, 1H, Ar), 7.73 (d, J' = 1.8 Hz, 1H Ar), 10.59 (s, 1H, exch D_2O, NH), 11.49 (s, 1H, exch D_2O, NH).

The same experiment, performed on 0.2 g (0.7 mmol) **19** in 5 mL acetonitrile to which 0.5 mL D_2O had been added, gave deuterated **18** to a large extent as demonstrated by the lower integration of the relevant NMR signals (scheme 5).

^1H NMR (DMSO-d_6): δ = 1.02–1.48 (m, 6H, cyclohexyl), 1.57–1.63 (m, 4H, cyclohexyl), 2.01 (m, 1H, cyclohexyl), 3.40 (d, J = 2.5 Hz, 0.26 H, CH), 4.19 (s, 0.34 H, SCH_2), 6.87 (d, J = 8.2 Hz, 1H, Ar), 7.65 (dd, J = 8.2 Hz, J' = 1.8 Hz, 1H, Ar), 7.73 (d, J' = 1.8 Hz, 1H Ar), 10.59 (s, 0.50 H, NH), 11.49 (s, 0.49H, NH).

Biology

Influence of compounds on Ca^{2+}-dependent ATPase activity of cardiac myofibrils
Canine cardiac myofibrils free of membrane contaminants were prepared using the method of Solaro and coworkers [37]. Ca^{2+}-dependent myofibrillar ATPase activity was determined at 21 °C by measuring the rate of release of inorganic phosphate (P_i). Assays were performed using the method described by Solaro and Ruegg [1] in reaction mixtures containing 0.6–0.7 mg/mL myofibrillar protein, 80 mM KCl, 20 mM imidazole, 3 mM $MgCl_2$, 2 mM Na_2ATP, 1 mM EGTA, and the desired amount of $CaCl_2$. The amount of $CaCl_2$ was varied between 0 and 0.9 mM and pCa (-log concentration of free Ca^{2+}) was computed using 2.514 x 106 M^{-1} as the apparent affinity of Ca^{2+} for EGTA at pH 7.0. Myofibrils were preincubated for 5 min in the presence of the studied compound, reaction was initiated by the addition of Na_2ATP, and after an incubation period of 12 min, reaction was quenched by the addition of an equal volume of ice-cold 10% aqueous trichloroacetic acid. Protein was pelleted by centrifugation and P_i was determined colorimetrically using a malachite green method [38].

Inhibition of the sarcoplasmic reticulum-bound low-K_m cAMP phosphodiesterase (SR-PDE)
The inhibition of the SR-PDE was performed using sarcoplasmic reticulum vesicles prepared from dog left ventricle as previously described by Jones and Cala [39]. Briefly, microsomes were prepared from canine ventricular tissue which is subjected to vigorous initial homogenization and to several centrifugation steps to remove nuclei, cell debris, and mitochondria. Free SR-vesicles were isolated by sucrose density gradient centrifugation of microsomes after selective Ca^{2+} oxalate loading.

The activity of cAMP-PDE was assayed by a radiochemical procedure at 30 °C in a medium containing 12 mM Tris-HCl, pH 7.7, 0.5 mM $MgCl_2$, 137 mM NaCl, 20 mM glucose and

1 μM [^{3}H]cAMP. This assay is a modification of the two-step technique of Thompson and co-workers [40] in which the substrate and products were separated using Dowex 1-X8 resin after AMP was fully converted to adenosine by 5'-nucleotidase. PDE reactions were initiated by adding sufficient enzyme to hydrolyze less than 25% of the substrate, and PDE activity was linear vs time during the assay. Test compounds had no significant effect upon the snake venom *(Crotalus atrox)* used to convert [^{3}H]AMP to [^{3}H]adenosine in the second step of the assay. Moreover, neither the recovery of [^{3}H]adenosine nor that of unreacted [^{3}H]cAMP were significantly affected by the test compounds (data not shown). PDE activity was determined in triplicate at ten inhibitor concentrations (10^{-9}–10^{-4} M) in order to generate inhibition curves. DMSO was utilized as solvent for PDE inhibitors, and controls were run to ensure that this solvent (1%, v/v) did not affect assay results. Results are expressed as IC_{50} values which were determined after linearization (Hill plot) of the mean inhibition curve obtained from at least three different SR-PDE preparations.

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