Differential analgesic activity of the enantiomers of atropine derivatives does not correlate with their muscarinic subtype selectivity

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Summary — The enantiomers of several tropic and *p*-substituted tropic acid esters related to atropine obtained by esterification under non-racemizing conditions after resolution of the corresponding racemic acids [(+)- and (-)-18, (+)- and (-)-19] are reported. They were tested in vitro on muscarinic subtype receptors and in vivo for their analgesic activity on mice. As in the case of the lead compound, R-(+)-hyoscyamine, these substances show enantioselectivity in analgesic tests, the eutomers being the R-(+) or R-(+)-*p*-substituted tropic acid derivatives. However, this property, which is a consequence of increased central release of ACh, seems unrelated to muscarinic subtype selectivity insofar as the compounds are unable to discriminate muscarinic subtype receptors. A possible explanation of these results which does not involve subtype selectivity is proposed, based on the recently developed concept of inverse agonism.

muscarinic antagonism / atropine derivative / subtype selectivity / enantiomer separation / enantioselectivity

Introduction

Over the past few years we have studied the particular behaviour of the enantiomers of atropine (R-(+)- and S-(-)-hyoscyamine, R-(+)- and S-(-)-1) on the hotplate test for analgesia. We found that at very low doses (1-10 µg kg⁻¹ sc) the R-(+) enantiomer is a fairly potent analgesic although not as efficacious as morphine, while the better studied S-(-) enantiomer, which is some 100-fold more potent as a muscarinic antagonist in most tissues, is at the same doses completely devoid of analgesic activity [1-3].

Preliminary structure-activity relationships showed that only minor modifications of the atropine molecule were necessary to maintain analgesic activity, but at the same time confirmed that such activity was a particular feature of the R-(+) enantiomers [1-3]. A body of evidence suggested that the analgesia produced by R-(+)-hyoscyamine and related compounds was cholinergic in nature [4–6] and due to an increased release of ACh in the CNS.

The simplest hypothesis that can be made about the mechanism of action of R-(+)-hyoscyamine is that its

analgesic effect is due to a specific blockade of the presynaptic muscarinic receptors controlling the release of ACh in the brain compared to the postsynaptic receptors, whose activation produces cholinergic analgesia and which are considered as belonging to the M_1 subtype [6]. It must be remembered in this respect that presynaptic muscarinic receptors are presumed to belong to the M_2 subtype even though the M_4 subtype may also be involved in some tissues [7, 8]. As a matter of fact it has been reported [9, 10] that the two M_2 muscarinic antagonists, AFDX-116 and methoctramine, are able to induce antinociception through a presynaptic mechanism of action since their analgesic effect is completely prevented by the ACh depletor hemicholinium-3. The increase in the pain threshold produced by AFDX-116 and methoctramine is also observed with the new M₂-selective antagonist tripitramine ([11]; and Ghelardini, pers commun).

Based on the above, we decided to study the pharmacological profile of a series of enantiomeric couples related to R-(+)- and S-(-)-hyoscyamine considering in particular their muscarinic subtype selectivity to check for a correlation between subtype selectivity and analgesic activity. To this end, we synthesized the enantiomers of several atropine derivatives and tested them on rabbit vas deferens (M_1),

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guinea pig atrium (M_2) and ileum (M_3) . Moreover, since R-(+)-hyoscyamine was able to discriminate the so far poorly characterized muscarinic receptor contracting the immature guinea pig uterus (M_u) [8], we included this tissue among those studied.

Chemistry

The structure of the compounds studied is reported in table I. Compounds 1 (racemate and enantiomers), 2 (racemate and enantiomers), 3 (racemate), 7 (racemate and S-(-) enantiomer), 9 (racemate), 11 (racemate and enantiomers), 12 (racemate), 13 (racemate), 15 (racemate and enantiomers) and 16 (racemate) have been previously described¹ [1-3, 12, 13]. The chemical and physical characteristics of the newly synthesized compounds are reported in table II.

Their synthesis (see scheme 1) required the resolution of the racemate of the corresponding tropic acids. While the tropic acid enantiomers were already known [14] and obtained by ourselves at high optical purity [1, 4, 8], the racemate of p-chloroand p-bromotropic acid (both synthesized according to Caldwell et al [15] as reported by Gualtieri et al [3]) had not been resolved before.

The resolution of *p*-chloro- and *p*-bromotropic acids $((\pm)-18$ and $(\pm)-19$) was performed by fractional crystallization of the diastereomeric salts using in sequence D-threo-2-amino-1-(4-nitrophenyl)-1,3-propanediol and quinine, quinine and quinidine, respectively. The optical purity of the enantiomers was assessed via ¹H-NMR of the Mosher esters [16] of the ethyl esters of S-(-)-18 and S-(-)-19. The ¹H-NMR of the diastereomeric ester mixture derived from the racemates 18 and 19 (R,R-20 + R,S-20 and R,R-21 + R,S-21 respectively) was compared with the ¹H-NMR of the compounds obtained from resolved enantiomers (RS-20 and RS-21). The results show (fig 1) that within the limits of the method $(\pm 5\%)$, the enantiomers appear to be optically pure. As can be seen from the corresponding optical rotation ($[\alpha]$) (see Experi*mental protocols*), the R-(+)-18 enantiomer is slightly less and the R-(+)-19 enantiomer slightly more pure than the corresponding enantiomers that were assessed. However, the method used does not allow quantification of this difference. In order to establish the absolute configuration of the enantiomers, circular dicroism of (+)- and (-)-18, (+)- and (-)-19 was also recorded and compared to the corresponding spectra of tropic acid enantiomers. Both (-)-18 and (-)-19
 Table I. Structure of the compounds studied as racemates and/or enantiomers.



Compound	R	Y		
1 ^{a,b,c} (Atropine)	Н	Α		
2 ^a	Н	В		
3 ^b	Cl	А		
4	Cl	В		
5	Br	Α		
6	Br	В		
7 b,d	Н	С		
8	Cl	С		
9 b	Н	D		
10	Cl	D		
11 ^{a,b}	Н	Е		
12 ^b	Cl	Е		
13°	Н	F		
14a	Cl	F		
15	Н	G		
16 ^b	Cl	G		

Compounds 1 (racemate and enantiomers), 2 (racemate and enantiomers), 3 (racemate), 7 (racemate and S-(-) enantiomer), 9 (racemate), 11 (racemate and enantiomers), 12 (racemate), 13 (racemate), 14 (racemate and enantiomers) and 16 (racemate) have already been described (see references indicated in a-d). aSee ref [1]; bsee ref [3]; csee ref [8]; dsee ref [12]; esee ref [13].

showed a negative Cotton effect similar to that shown by S-(-) tropic acid; on the contrary, (+)-18 and (+)-19 showed a positive Cotton effect as R-(+) tropic acid allowing an obvious correlation of CD curves with the absolute configuration.

Finally, the enantiomers were esterified with a suitable aminoalcohol (see table I) under non-racemizing conditions [17]. When esterified with α -tropanol, the derivatives were also transformed into the corresponding methyl iodides by treatment with CH₃I.

Pharmacology

The analgesic activity of the compounds was tested on mice with the hot-plate test [18]. Analgesic activity

¹The chemical and physical characteristics of *S*-(-)-7 were reported because until now, as far as we know, these were only obtained by extraction from plants or by separation of noratropine, see [12].

Compound	$[\alpha]_D^{20a}$	Saltf	Mp(°C)	Formula ^j
S-(-)- 3	-7.0 ^b	HCl	167–170g	$C_{17}H_{23}Cl_2NO_2$
<i>R</i> -(+)- 3	+7.0 ^b	_	99–102 ^b	$C_{17}H_{22}ClNO_3$
(±)- 4	_	Methyl iodide	216–218°	C ₁₈ H ₂₅ ClINO ₃
S-(-)- 4	-4.9°	Methyl iodide	211-213°	C ₁₈ H ₂₅ ClINO ₃
<i>R</i> -(+)- 4	+5.0°	Methyl iodide	212–214°	C ₁₈ H ₂₅ ClINO ₃
(±)-5	<u> </u>	Oxalate	65–68 ⁱ	$C_{19}H_{24}BrNO_7$
S-(-)- 5	-4.2 ^{b,d}	_	48-50 ^b	$C_{17}H_{22}BrNO_3$
R-(+)- 5	+4.6 ^{b,d}	_	49–52 ^b	$C_{17}H_{22}BrNO_3$
S-(-)-6	-1.8 ^{c,e}	Methyl iodide	216–218°	$C_{18}H_{25}BrINO_3$
R-(+)- 6	+2.0 ^{c,e}	Methyl iodide	218–219°	$C_{18}H_{25}BrINO_3$
S-(-)-7	-18.2 ^b	HCl	132 decg	$C_{16}H_{22}CINO_3$
R-(+)-7	+18.4 ^b	HCl	134 decg	$C_{16}H_{22}CINO_3$
(±)- 8	_		112–114 ^b	$C_{16}H_{20}CINO_3$
S-(-)- 8	-6.5 ^b	HCl	75–78s	$C_{16}H_{21}Cl_2NO_3$
R-(+)- 8	+6.8 ^b	_	114–116 ^b	$C_{16}H_{20}CINO_3$
S-(-)- 9	-14.6 ^b	_	78–80 ^b	$C_{18}H_{25}NO_{3}$
R-(+)- 9	+14.8 ^b	_	79–81 ^b	$C_{18}H_{25}NO_{3}$
(±)-10	_	_	105–106ь	$C_{18}H_{24}CINO_3$
S-(-)- 10	-8.2 ^b	_	100-102ь	$C_{18}H_{24}ClNO_3$
<i>R</i> -(+)-10	+8.6 ^b	_	102–104 ^b	$C_{18}H_{24}ClNO_3$
S-(–)- 12	-8.2 ^b	_	h	$C_{15}H_{20}ClNO_3$
<i>R</i> -(+)- 12	+8.2 ^b	_	h	$C_{15}H_{20}CINO_3$
(±)-13	-	_	100–101 ^b	$C_{18}H_{25}NO_{3}$
S-(-)- 13	-11.0 ^b	HCl	150–152g	$C_{18}H_{26}CINO_3$
<i>R</i> -(+)- 13	+11.2 ^b	HCl	148-151g	C ₁₈ H ₂₆ ClNO ₃
(±)-14	_	Oxalate	68–70 dec ⁱ	$C_{20}H_{26}CINO_7$

Table II. Chemical and physical characteristics of the newly synthesized compounds.

was expressed by the two parameters, ED_{50} and efficacy, with respect to morphine 8 mg/kg sc, according to previously reported experiments [3]. The cholinergic origin of the analgesic action was checked by reverting it with atropine (5 mg/kg ip).

Muscarinic antagonism was evaluated by functional studies performed on rabbit vas deferens (M_1) , guinea pig atrium (M_2) and ileum (M_3) using McN-A-343, carbachol and ACh as agonists, respectively. Guinea pig immature uterus (M_u) was also included in the test (carbachol as agonist).

Results are expressed as pA_2 for the reference compounds (±)-1, R-(+)-1 and S-(-)-1 and for S-(-)-11, and the M_u value of R-(+)-2 and as pK_b for the other compounds.

 pA_2 's were calculated according to Schild [19], constraining the slope of the curve to -1.0 as required

by theory [20]. It was always verified that the experimental data generated a line whose derived slope was not significantly different from unity.

 pK_b 's $(-\log K_b)$ were obtained at a given concentration of antagonist from the Van Rossum equation: lg (DR-1) = lg [Ant]- $lg K_b$ [21]. For both pA_2 and pK_b evaluations, each concentration was tested from four to seven times. No reduction in the maximum effect was observed at the doses of antagonist used.

Results

The results of the hot-plate test are reported in table III and V. In all cases the R-(+) enantiomer was active and the S-(-) isomer inactive, the only exception being compound S-(-)-9, whose analgesia is, however,

^aUnless otherwise stated all measurements were carried out in abs ethanol; ^bas free base; ^cas methyl iodide; ^dCHCl₃; ^eMeOH; ^fall the salts were crystallized from an ethanol/an ether mixtures; ^gas hydrochloride; ^hoil; ⁱas oxalate; ⁱthe compounds were analyzed for C, H, N as salts when done, or as free base.



Scheme 1. D-threo-2-amino-1-(4-nitrophenyl)-1,3-propanediol and quinine for **17** [14] and **18**; quinine and quinidine for **19**. b) CH₃COCl; c) SOCl₂; the suitable base (see table I) as hydrochloride; d) conc HCl; e) EtOH, HCl; f) $(F_3 - C_{CCl})$

о́сн



Fig 1. ¹H-NMR of the Mosher esters of *p*-Cl and *p*-Br tropic acid ethyl sters. Here the signal corresponding to the diagnostic methylene protons is amplified. The results show that, within the limits of the method, the enantiomers are pure.

non-cholinergic in nature. The enantiomers of racemic compounds 14, which also shows a non-cholinergic analgesia, and 16, which is inactive, were not studied further.

The results of functional studies are reported in tables III–V together with the eudismic ratio (Eu) of each couple for each tissue. The eudismic ratio represents the ratio between the potency of the less potent enantiomer (distomer: Dis) and that of the most potent enantiomer (eutomer: Eu). It is a measure of the enantioselectivity of binding to the receptor. Compounds **1–6** were tested on all tissues studied and their antimuscarinic activity is reported in table III.

Since all compounds reported in table III did not discriminate between M_1 , M_2 and M_3 receptors (see table IV), to save time and animals we decided to limit the testing of the other derivatives only to M_2 and M_u receptors. As a matter of fact, the only relevant feature of the data shown in table III is that the enantiomers of compounds 1, 2 and 3, which are active as analgesics, also show higher affinity for the muscarinic receptor of immature guinea pig uterus.

Table III.	Functional	muscarinic	antagonism	and analgesic	activity ^a of	p-substituted	atropine	analogs	1-6.
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Compound			Functional muscarinic antagonism						Analgesic activity	
	<i>M</i> ₁ b	Euf	M_2^{c}	Euf	M_{j} d	Euf	M_u^{e}	Euf	<i>ED</i> ₅₀ (<i>mg/kg</i>) ^g	Analg efficacy (%) ^h
(±)-1 S-(-)-1	9.46 9.04 (0.07)	100	8.92 8.95 (0.01)	50	9.0 9.04 (0.03)	144	9.50 (0.13) 8.59 (0.01)	0.1 ⁱ	7.0 (6.5–7.4)•10-4 Inactive	42
<i>R</i> -(+)-1	7.05 (0.05)	100	7.25 (0.04)	20	6.88 (0.05)	1	9.56 (0.01)	0.1	4.0 (3.7-4.2)-10-4	41
S-(-)- 3	8.52 (0.06)	2	8.62 (0.11)	10	9.48 (0.10)	7	8.73 (0.05)	0 4i	6.0 (5.1–6.6)•10 ⁻⁴ Inactive	37
<i>R</i> -(+)- 3	8.20 (0.12)	2	7.64 (0.11)	10	8.64 (0.08)	,	9.11 (0.07)	0.1	7.1 (6.1–7.7)•10–4	47
(±)-5 S-(-)-5	8.38 (0.11) 8.87 (0.13)	0	8.45 (0.10) 8.97 (0.09)	4	8.79 (0.12) 8.46 (0.09)	2	8.81 (0.07) 8.97 (0.03)	4	21.3 (15.4–26.1)•10 Inactive	-3 42
<i>R</i> -(+)- 5	7.98 (0.14)	8	8.34 (0.15)	4	7.98 (0.04)	5	8.35 (0.08)	4 10.2	10.2 (6.2–13.3)•10-	3 31
S-(-)-2	10.0 (0.11)	33	10.0 (0.12)	24	9.49 (0.07)	13	8.35 (0.12)	0.08	Inactive ^j	
<i>R</i> -(+)- 2	8.48 (0.06)	00	8.62 (0.09)		8.37 (0.06)		9.47 (0.06)		6.4 (5.0–7.5)•10 ^{-6^j}	42
(±)- 4 S-(-)- 4	8.88 (0.08) 9.39 (0.06)	11	8.72 (0.11) 8.80 (0.10)	1	8.86 (0.06) 9.02 (0.08)	1	8.67 (0.06) 9.21 (0.13)	2	9.4 (8.3-10.2)•10-6- Inactive ¹	^j 37
<i>R</i> -(+)- 4	8.35 (0.08)	11	8.81 (0.10)	I	8.91 (0.08)	1	8.84 (0.02)	2	17.8 (15.4–20.2)•10-	- ^{6j} 39
S-(-)-6	9.00 (0.04)	5	9.17 (0.06)	4	9.27 (0.05)	3	9.24 (0.07)	3	Inactive	
<i>R-</i> (+)- 6	8.34 (0.09)	5	8.53 (0.05)	-	8.81 (0.06)	5	8.77 (0.06)	5	22.7(18.2-26.8)•10-	^{6j} 40

^aUnless otherwise indicated, antagonism is expressed as pK_b ($-lg K_b$), calculated according to Van Rossum [21] at only one concentration (10⁻⁸ M). Each concentration was tested from four to seven times. The SEs are in brackets. The concentration was changed for the following compounds: 10⁻⁷ M for compound R-(+)-3 (M₁); 3 x 10⁻⁸ M for compounds S-(-)-3 (M₁), R-(+)-3 (M₂), (±)-5 (M₂), R-(+)-5 (M₁ and M₃), R-(+)-6 (M₂); 10⁻⁹ M for compound S-(-)-2 (M₃). The data reported for S-(-)-1, R-(+)-1 and R-(+)-2 (M_u), are pA₂ calculated according to Schild [19] (see refs [28–31]); ^brabbit vas deferens; ^cguinea pig atrium (Force); ^dguinea pig ileum; ^eguinea pig uterus; ^feudismic ratio is the antilog of the difference between the pA₂ (or pK_b) values for the eutomer and the diastomer; ^ginjected sc unless otherwise stated; ^breferred to 8 mg/kg sc of morphine taken as a reference (see *Experimental protocols*); ⁱby definition, Eu is larger than 1. Here the reverse is reported to emphasize the inversion of enantioselectivity; ^jinjected intracerebroventricularly.

Compound			Selectivity ratios ^a					
	$\overline{M_2/M_1}$	$M_{\mathcal{J}}M_{1}$	M_{i}/M_{i}	M_{J}/M_{2}	M_u/M_2	M_u/M_3		
(±)-1	0.3	0.3	1	1	4	3		
S-(-)-1	0.8	1	0.3	1	0.4	0.3		
<i>R</i> -(+)-1	2	0.7	323	0.4	204	479		
S-(-)- 3	1	9	2	7	1	0.2		
<i>R</i> -(+)- 3	0.3	3	8	10	30	3		
(±)-5	1	3	3	2	2	1		
S-(-)-5	1	0.4	1	0.3	1	3		
<i>R</i> -(+)-5	2	1	2	0.4	1	2		
S-(-)- 2	1	0.3	0.02	0.3	0.02	0.07		
<i>R</i> -(+)- 2	1	1	10	0.6	7	13		
(±)- 4	0.7	1	0.6	1	1	0.6		
S-(-)- 4	0.3	0.4	0.7	2	3	2		
<i>R</i> -(+)- 4	3	4	3	1	1	0.9		
S-(-)-6	2	2	2	1	1	1		
<i>R</i> -(+)-6	2	3	3	2	2	0.9		

Table IV. Selectivity ratios of compounds 1-6.

^aThe selectivity ratio is the antilog of the difference between the pA_2 (or pK_b) values of the corresponding tissues.

The antimuscarinic potency of compounds 7-16 is reported in table V.

Discussion and conclusion

As far as analgesic activity is concerned, the results obtained with R-(+)- and S-(-)-hyoscyamine [1-3] have been confirmed in the series of analogues synthesized and studied in the present work.

With the exception of compounds 13 and 16 (which are inactive as analgesics) and 14 whose analgesia is not cholinergic, the R-(+)-enantiomers consistently showed high analgesic potency, while the corresponding S-(-) enantiomers were completely devoid of analgesic activity. S-(-)-9 would appear to be an exception, but its analgesic activity is non-cholinergic.

It is interesting that the quaternary ammonium salts 2, 4 and 6 (both enantiomers and racemates) which are inactive when administered peripherally (data not shown) showed the same profile of the corresponding tertiary amines 1, 3 and 5 if injected intracerebroven-tricularly thus confirming the central origin of the analgesic action [5].

As regards their cholinergic activity, all compounds are potent muscarinic antagonists, their affinity ranging from $pK_b = 10.00 (S - (-) - 2 \text{ on rabbit vas defe$ $rens and guinea pig heart) to <math>pK_b = 6.31 (R - (+) - 15 \text{ on}$ the same preparation). The enantioselectivity of the racemic couples as represented by the eudismic ratios (see tables III and V) is lower for the derivatives compared to that of the parent compounds R-(+) and S-(-)-1. It can be excluded that this is due to incomplete resolution of the enantiomers, since the starting materials (*p*-substituted tropic acids) were well resolved (see *Experimental protocols*), the esters were obtained under non-racemizing conditions, and the final compounds did not racemize in physiological solution, as verified on compounds R-(+)-1 and R-(+)-2 over a period of 1 week.

As mentioned in the Introduction, it has been shown that the analgesic activity of R-(+)-1 is due to the amplification of central cholinergic transmission [4–6]. Even if as shown for R-(+)-1 ACh release has not been proven directly, it is very likely that this is also the reason for the analgesic activity of the compounds studied in the present work. Since heteroreceptors known to control ACh release are apparently not involved in the activity of R-(+)-1 [4], a reasonable explanation for its analgesic activity would be that of a selective blockade of presynaptic muscarinic receptors controlling the release of ACh (autoreceptors). These receptors in most cases seem to belong to the M_2 subtype, even if suggestions on the involvement of the M_4 subtype have been advanced [7]. However, R-(+)-1 does not discriminate M_2 [4-8] or M_4 receptors [4]; on the contrary, it shows a specific recognition of the muscarinic receptor contracting immature guinea pig uterus [8].

Even for the analogue studied in the present work, subtype selectivity was found to be very low for all three muscarinic receptor subtypes tested (M_1, M_2) and M_3), with the exception of the muscarinic receptor contracting guinea pig uterus (M_u). In fact, as previously reported [8], R-(+)-hyoscyamine (R-(+)-1) is able to discriminate this so far poorly characterized receptor from the M₁-M₃ counterparts. It is also remarkable that in this tissue the eudismic ratio is inverted, R-(+)-1 being the eutomer (most potent enantiomer) and S(-)-1 the distomer (less potent enantiomer). As far as the eudismic ratio is concerned, the behaviour of the enantiomers of 1 on the receptor contracting the guinea pig uterus is maintained in its methyl iodide 2 and in the *p*-chloro analogue 3. However, as can be seen from table IV, the discriminating properties for the M_{μ} receptor of the R-(+)enantiomers of these compounds are rather less impressive than those of R-(+)-1. All other compounds studied did not show any interesting subtype selectivity, either on M_1-M_3 or on M_u receptors.

One of the referees noticed that all the enantiomers in table III active as analgesics showed an affinity for the M_{μ} receptors which was higher than that for M_{μ}

Compound	F	Functional mus	Analgesic activity			
	M ₂ ^c	Euť	M_{u}^{e}	Euf	ED ₅₀ (mg/kg) ^g	Analgesic efficacy (%) ^h
(±)-7 S-(-)-7	9.55 (0.14)	11	8.59 (0.15)	6	5 (3.8–6.0) x 10 ⁻³ Inactive	35
<i>R</i> -(+)-7	8.49 (0.11)	11	7.78 (0.09)	0	4.7 (3.5–6.2) x 10-4	51
S-(-)- 8	7.06 (0.16)	4	7.78 (0.06)	3	Inactive	
<i>R</i> -(+)- 8	6.50 (0.10)		7.25 (0.08)		6.9 (5.8–8.1) x 10-4	42
(±)-9 S-(-)-9	8.90 (0.11)	10	8.76 (0.10)	5	7.3 (5.3–8.6) x 10 ⁻³ 55.7 (43–74) x 10 ⁻³	33 46 ⁱ
<i>R</i> -(+)- 9	7.93 (0.13)	10	8.05 (0.04)	5	29 (21–40) x 10 ^{–3}	29
(±)-10 S-()-10	8.15 (0.15) 8.38 (0.04)	5	7.73 (0.08) 8.63 (0.06)	7	92 (75–105) x 10 ⁻³ Inactive	32
<i>R</i> -(+)- 10	7.66 (0.02)	5	7.76 (0.05)	,	92.6 (87.7–96.3) x 10-	3 30
<i>S</i> -(–)- 11	8.21 (0.06)	10	8.70 (0.10)	28	Inactive	
<i>R</i> -(+)- 11	7.20 (0.08)		7.26 (0.13)		4.1 (3.5–5.0)	41
(±)-12 S-(-)-12	7.92 (0.04) 8.60 (0.06)	14	7.65 (0.11) 8.31 (0.07)	11	6.6 (4.3–8.9) Inactive	26
<i>R</i> -(+)- 12	7.49 (0.12)		7.26 (0.03)		8.3 (7.2–9.6)	39
(±)-13 S-(-)-13	9.51 (0.09)	10	9.30 (0.08) 9.31 (0.08)	11	Inactive	
<i>R</i> -(+)-13	8.51 (0.08)	10	8.27 (0.06)		Inactive	
(±)-14 S-(-)-15	8.38 (0.13) 7.02 (0.17)	5	8.26 (0.15) 6.77 (0.07)	1	2.8 (2.0–3.5) Inactive	64.5 ⁱ
<i>R</i> -(+)- 15	6.31 (0.13)	5	6.75(0.12)	1	2.8 (1.9-3.6)	38.7
(±)-16	6.89 (0.03)		6.78 (0.12)		Inactive	

Table V. Functional muscarinic antagonism and analgesic activity^a of ester-modified atropine analogs (7-16).

^aUnless otherwise indicated, antagonism is expressed as pK_b (-lg K_b), calculated according to Van Rossum [21] at only one concentration (10⁻⁷ M). Each concentration was tested from four to seven times. The SE's are in brackets. The concentration has been changed for the following compounds: 10⁻⁸ M for compounds: S-(-)-7 (M₂), S-(-)-9 (M_u), S-(-)-10 (M_u), S-(-)-12 (M_u), (±)-13 (M_u), S-(-)-13 (M₂), R-(+)-13 (M₂), (±)-14 (M₂, M_u); 3 × 10⁻⁸ M for compounds: (±)-10 (M₂, M_u), S-(-)-12 (M₂), R-(+)-13 (M_u); 3 × 10⁻⁷ M for compounds: R-(+)-8 (M₂, M_u), S-(-)-9 (M₂). R-(+)-11 (M₂), R-(+)-12 (M₂); 10⁻⁶ M for compounds: S-(-)-15 (M₂, M_u). R-(+)-15 (M₂, M_u), (±)-16 (M₂, M_u). The data reported for S-(-)-11 (M₂, M_u), are pA₂ calculated according to Schild [19] (see [29, 31]); ^{c,f,g,h}see the corresponding footnotes in table III; inon cholinergic analgesia.

receptors, and suggested that this difference could account for their antinociceptive action if the presynaptic receptor in CNS was of the M_u subtype.

As a matter of fact, we also noticed this trend. However, as their differences were rather small in several cases (see R-(+)-4, R-(+)-5, and R-(+)-6), and given the present uncertainty regarding the nature of the M_u receptor and its equivalence with any of the muscarinic subtypes present in the CNS, this indicated that we should be cautious and avoid speculation. On the other hand, the results reported in table V show that the analgesic properties of R-(+)-enantiomers are neither related to high affinity nor to selectivity toward M_u receptors.

Taken together, the results obtained in this study seem to exclude a correlation between analgesic activity and muscarinic subtype selectivity, even if the high affinity and selectivity of R-(+)-1 for the receptor contracting the guinea pig uterus casts some doubt on the nature of this muscarinic subtype [8].

A possible explanation that does not involve subtype selectivity is based on the recently developed concept of inverse agonism [22] (also referred to as negative antagonism [23] and negative efficacy antagonism [24]). As a matter of fact, Tucek [23] recently reported that atropine behaves as a negative antagonist on the muscarinic receptor of transfected CHO cells and rat cardiomyocytes.

It can be hypothized that presynaptic receptors involved in R-(+)-1 activity display constitutive (spontaneous) activity. In other words, muscarinic autoreceptors whatever the subtype involved could be present in two states: active and inactive. In the absence of ACh, the equilibrium of the two states would favor the active state, thus maintaining the system in a constitutive inhibition.

R-(+)-hyoscyamine at low doses by preferentially stabilizing the inactive form of the receptor would shift the equilibrium toward the inactive state. As a consequence, the removal of constitutive inhibition by R-(+)-hyoscyamine would result in the increase of ACh release. Higher doses of R-(+)-hyoscyamine would always be able to discriminate between the two states, but the effect on ACh release would be obscured by postsynaptic receptor blockade.

The S-(-) enantiomer (S-(-)-1), although having more affinity toward known and pharmacologically characterized receptors (M_1, M_2, M_3) would not discriminate between the two states and would be unable to modify the constitutive inhibition of ACh release. Studies are underway to check this hypothesis.

In conclusion: (a) the analgesic activity of R-(+)hyoscyamine is also shown by the R-(+) enantiomers of the analogues synthesized and studied in the present work; (b) this property which is the consequence of increased central release of ACh and could possibly be due to selective blockade of muscarinic presynaptic receptors cannot be explained by subtype selectivity, as the compounds studied were unable to differentiate M_2 from M_1 and M_3 receptors; (c) the discriminating properties of R-(+)-1, R-(+)-2 and R-(+)-3 toward the muscarinic receptor contracting guinea pig uterus (M_u) do not seem to be related to their analgesic activity; (d) the concept of inverse agonism seems to be a reliable working hypothesis to explain the analgesic activity of R-(+)-1 and its R-(+) analogs.

Experimental protocols

Chemical synthesis

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin– Elmer 681 spectrophotometer in a Nujol mull for solids and neat for liquids. Unless otherwise stated, NMR spectra were recorded on a Gemini 200 spectrometer. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm, Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm, Merck). Yields are given after purification unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within $\pm 0.4\%$ of the theoretical values. Optical rotation was measured at a concentration of 1 g/100 mL (c = 1), unless otherwise stated, with a Perkin–Elmer 241 polarimeter (accuracy $\pm 0.002^\circ$). CD was measured at a concentration of 1 mg/ mL (ethanol, 1 cm and 1 mm cells) with a Jasco 500 C spectropolarimeter.

General method for the synthesis of racemic and chiral esters 1, 3, 5, 7–16

The aminoalcohols used as starting materials are commercially available or were obtained according to methods given in the literature². Their esters can be obtained following this general procedure:

Five mmol of the appropriate tropic acid were dissolved in 5 mL acetyl chloride and the mixture refluxed for 2 h. Excess reagent was eliminated and the oily residue stirred at 60 °C for 2.5 h with 5 mL SOCl₂. The excess SOCl₂ was removed under reduced pressure, the residue dissolved in cyclohexane and the solvent eliminated under vacuum; this operation was repeated three times.

The oily acid chloride obtained was treated with 5 mmol hydrochloride of the appropriate aminoalcohol and the mixture stirred under nitrogen for 2 h at 80 °C. When necessary, the solubilization was aided by 5 mL ethanol-free CHCl₃. When cool, the mixture was dissolved in 3 mL water, treated with 1.5 mL conc HCl and stirred at room temperature for 24 h. After alkalinization with 10% Na₂CO₃, the solution was extracted with CHCl₃ and evaporated to give an oil from which

² α -Tropanol, *N*-methylpiperidinol and *N*,*N*-diethylaminoethanol are commercially available; α -nortropanol was obtained according to Kraiss [25], *N*-ethyl and *N*-benzyl- α -nortropanol were obtained according to Berthold [26], *N*-methyl- α -granatanin-3-ol was obtained according to Bryant [27].

minor impurities were removed by silica gel column chromatography using CHCl₃/petroleum ether/absolute ethanol/NH₄OH 340:60:65:8 as eluent.

The products are reported in tables I and II. When necessary they were transformed into salts as reported in the tables.

General method for the synthesis of methyl iodides 2, 4, 6

One mmol of the appropriate ester was dissolved in 20 mL anhydrous ether, treated with an excess of CH_3I and left overnight at room temperature in the dark. The white crystals were collected and recrystallized from absolute ethanol. The characteristics of the compounds are reported in tables I and II.

Resolution of racemic 4-chlorotropic acid 18

Racemic *p*-chlorotropic acid [15], 9.6 g (47.8 mmol) were dissolved in 96 mL water and 10.12 g (47.8 mmol) D-(–)-threo-2-amino-1-(4-nitrophenyl)-1,3-propanediol added. The crystals formed at room temperature were collected and recrystallized several times from abs ethanol to constant rotation, $[\alpha]_D^{20} = -25.8$ (c = 0.5, abs EtOH), mp = 151–153 °C. The salt was dissolved in hot water and the solution alkalinized with conc NH₄OH. After standing for 1 h in ice–water, the aminodiol base was filtered off. The concentrated solution was then acidified with HCl conc to pH = 1, and cooled in ice–water, where-by (–)-4-chlorotropic acid was separated (2.1 g, 10.5 mmol), $[\alpha]_{D}^{20} = -51.6$ (c = 0.5, abs EtOH), mp = 152–154 °C, yield 43.7%.

The mother liquors were evaporated to dryness and the residue was treated in the same way, yielding 6.65 g residue acid (33.1 mmol) which was dissolved in 100 mL ethanol and treated with 10.75 g (33.1 mmol) quinine. The crystals formed at room temperature were collected and recrystallized several times from ethanol to constant rotation, $[\alpha]_{2}^{D0} = -100.6$ (c = 0.5, abs EtOH), mp = 165–167 °C. The salt was dissolved in the minimum amount of water, the solution acidified with 2 N H₂SO₄ and extracted with chloroform to give 3.74 g (18.6 mmol) of the acid, $[\alpha]_{2}^{D0} = +50.4$ (c = 0.5, abs EtOH), mp = 151–154 °C, yield 77.9%.

Resolution of racemic 4-bromotropic acid 19

Racemic *p*-bromotropic acid [15], 3.90 g (15.9 mmol) were dissolved in 15 mL abs ethanol and 5.16 g (15.9 mmol) quinine added. The crystals formed at room temperature were collected and recrystallized several times from abs ethanol to constant rotation, $[\alpha]_{D}^{20} = -102$ (c = 0.5, abs EtOH), mp = 179–180 °C. The salt was dissolved in the minimum amount of water, the solution acidified with 2 N H₂SO₄ and extracted with chloroform to give 1.26 g (5.14 mmol) of the acid, $[\alpha]_{D}^{20} = -41.8$ (c = 0.5, abs EtOH), mp = 141–143 °C, yield 64.6%.

The mother liquors were evaporated to dryness and the residue was treated in the same way, yielding 2.56 g (10.4 mmol) of the residue *p*-bromotropic acid which was dissolved in 22 mL ethanol and treated with 3.6 g (10.4 mmol) quinidine. The crystals formed at room temperature were collected and recrystallized several times from ethanol to constant rotation. $[\alpha]_{20}^{20} = +156.6$ (c = 0.5, abs EtOH), mp = 166–173 °C. The acid recovered as described above (1.04 g, 4.2 mmol) had $[\alpha]_{20}^{20} = +43.4$ (c = 0.5, abs EtOH), mp = 142–144 °C, yield 53.3%.

Optical purity of 4-chloro and 4-bromotropic acid: synthesis of (R,S + R,R)-20, R,S-(-)-20, (R,S + R,R)-21 and R,S-(-)-21

The optical purity of the two enantiomers of 4-chloro and 4bromotropic acid was checked via the ¹H-NMR of the Mosher ester of the racemate [(RR) and (RS)] and that of one single isomer (RS). Within the limits of this technique, the two enantiomers were found to be pure. R-(+)- α -Methoxy- α -trifluoromethylphenylacetic acid (R-(+)-MTPA), 1.13 g (4.82 mmol) and 0.012 mg NaCl were dissolved in 4 mL SOCl₂. The mixture was refluxed for 24 h. The excess SOCl₂ was removed under reduced pressure, the residue dissolved in cyclohexane and the solvent eliminated under vacuum; this operation was repeated twice, yielding 1.04 g (4.11 mmol) of the corresponding acid chloride.

The oily acid chloride obtained was treated with an equimolar quantity of the ethyl ester of the appropriate tropic acid ((\pm)-4-chloro, (-)-4-chloro, (\pm)-4-bromo, (-)-4-bromo) in anhydrous CCl₄ (chloro derivatives) or CH₂Cl₂ (bromo derivatives), and the mixture stirred under nitrogen at room temperature for 24 h. After alkalinization with a saturated solution of NaHCO₃, the solution was extracted with diethyl ether and evaporated to give an oil from which impurities were removed by silica gel column chromatography using CHCl₃ as eluent.

(*R*,*S* + *R*,*R*)-20. IR v 1740, 1750 (COO) cm⁻¹; ¹H-NMR ∂ (CDCl₃) 1.20 (t, 3H, *J* = 7.2 Hz, CH₃), 3.45 (s, 3H, OCH₃), 3.95–4.20 (m, 3H, CH-CO and CH_2CH_3), 4.51–4.61 (m, 1H, methylenic H), 4.73–4.83 (m, 1H, methylenic H), 7.20–7.40 (m, 9H, aromatics) ppm. Anal (C₂₁H₂₀ClF₃O₅): C, H, Cl.

R,*S*-(-)-20. IR v 1740, 1750 (COO) cm⁻¹, ¹H-NMR ∂ (CDCl₃) 1.19 (t, 3H, *J* = 7.2 Hz, CH₃), 3.46 (s, 3H, OCH₃), 3.95–4.18 (m, 3H, CH-CO and *CH*₂CH₃), 4.58 (dd, 1H, ³*J* = 6.8 Hz, *J*_{gem} = 11 Hz, methylenic H), 4.89 (m, dd, 1H, ³*J* = 8.4 Hz, *J*_{gem} = 11 Hz, methylenic H), 7.24–7.38 (m, 9H, aromatics) ppm. Anal (C₂₁H₂₀ClF₃O₅): C, H, Cl.

(*R*,*S* + *R*,*R*)-21. IR v 1740, 1750 (COO) cm⁻¹, ¹H-NMR ∂ (CDCl₃) 1.18 (t, 3H, J = 7.2 Hz, CH₃), 3.45 (s, 3H, OCH₃), 3.92–4.18 (m, 3H, CH-CO and *CH*₂CH₃), 4.52–4.61 (m, 1H, methylenic H), 4.72–4.83 (m, 1H, methylenic H), 7.12–7.51 (m, 9H, aromatics) ppm. Anal (C₂₁H₂₀BrF₃O₅): C, H.

R,*S*-(-)-21. IR v 1740, 1750 (COO) cm⁻¹, ¹H-NMR ∂ (CDCl₃) 1.18 (t, 3H, *J* = 7.2 Hz, CH₃), 3.45 (s, 3H, OCH₃), 3.93–4.16 (m, 3H, CH-CO and *CH*₂CH₃), 4.67 (dd, 1H, ³*J* = 6.8 Hz, *J*_{gem} = 11 Hz, methylenic H), 4.78 (m, dd, 1H, ³*J* = 8.2 Hz, *J*_{gem} = 11 Hz, methylenic H), 7.14–7.47 (m, 9H, aromatics) ppm. Anal (C₂₁H₂₀BrF₃O₅): C, H.

Pharmacological evaluation

Animals

Male guinea pigs (200–300 g), female immature guinea pigs (150–200 g), male New Zealand white rabbits (2.50–3 kg), and male Swiss–Webster mice (22–28 g) were used. The animals were kept at 23 ± 1 °C, with a 12 h light/dark cycle, light at 7 am, with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community Council.

In vitro pharmacological evaluation

General considerations

Male guinea pigs, female immature guinea pigs and male New Zealand white rabbits were killed by cervical dislocation and the organs were set up under the appropriate tension (see below) in a 13-mL organ bath containing physiological salt solution (PSS) kept at an appropriate temperature (see below) and aereated with 5% CO_2 -95% O_2 . Dose-response curves were constructed by addition of the agonist (cumulative curves in the case of rabbit vas deferens and guinea pig atrium).

The concentration of agonist in the organ bath was increased approximately three-fold each step, each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Tissues were incubated with the antagonist for 1 h and a new dose-response curve to the agonist was obtained. Contractions were recorded by means of a force transducer connected to a single channel recorder (U Basile).

Muscarinic antagonism was evaluated on rabbit vas deferens (M_1) [28], guinea pig atrium (Force, M_2) [29], guinea pig ileum (M_3) [30] and immature guinea pig uterus (M_u) [31], with some small modifications with respect to the cited references.

All drugs that were available as free base were dissolved in an amount of 0.1 M HCl exceeding the stechiometric requirement by +10% and then diluted with saline.

Rabbit-stimulated vas deferens

The surrounding tissue was carefully removed from the vasa deferentia which were then divided into four segments, two prostatic portions of 1 cm and two epididymal portions approximately 1.5 cm long. The four segments were mounted under 0.75 g tension in PSS of the following composition (mM): NaCl (118.4), KCl (4.7), CaCl₂ (1.8), MgCl₂ (0.6), KH₂PO₄ (1.18), NaHCO₃ (25); glucose (11.1); 10⁻⁶ yohimbine was included to block alpha₂-adrenoceptors. The solution was maintained at 32 °C and tissues were stimulated through platinum electrodes by square-wave pulses (2 ms, 0.1 Hz, 10–30 V). Contractions were measured isometrically after tissues had been equilibrated for 1 h, then a cumulative dose–response curve for the inibitory effect of McN-A-343 was plotted.

Guinea pig stimulated left atrium

The heart was rapidly removed and the left atrium was excised and mounted under 1 g tension in PSS of the following composition (mM): NaCl (137), KCl (2.7), CaCl₂ (1.8), MgCl₂ (1.05), NaH₂PO₄ (0.42), NaHCO₃ (11.9); glucose (5.6). The solution was maintained at 30 °C and stimulated through platinum electrodes by square-wave pulses (1 ms, 1 Hz, 4–10 V). Inotropic activity was recorded isometrically. Tissues were equilibrated for 1 h and a cumulative dose–response curve to carbachol was plotted.

Guinea pig ileum

Two-cm long portions of terminal ileum were removed at about 5 cm from the ileum-cecum junction and mounted under 1 g tension in PSS (the same as that used for atria) at 37 $^{\circ}$ C. Tension changes were recorded isotonically. Tissues were equilibrated for 1 h and a dose-response curve to acetylcholine was obtained.

Guinea pig uterus

Uterine horns were divided into four portions and mounted under 1 g tension in PSS of the following composition (mM): NaCl (154), KCl (5.63), CaCl₂ (0.54), MgCl₂ (0.95), NaHCO₃ (5.95); glucose (2.78). The preparations were maintained at 30 °C and after a 1-h equilibration period, isotonic contractions to carbachol were recorded. Initially the tissues were exposed to a single concentration of carbachol (3 µmol/L) to check the responsiveness to the agonist and a dose-response curve for carbachol was obtained.

In vivo pharmacological evaluation

General considerations

All drugs that were available as free base were dissolved in 0.1 M HCl exceeding +10% the stechiometric requirement and

then diluted with saline. Drug concentrations were prepared so that the necessary dose could be injected in a vol of 10 mg/kg by both sc and ip routes. To ascertain the exact site of icv injection, some mice were injected icv with 5 μ L 1:10 diluted Indian ink, and their brains examined macroscopically after sectioning.

Analgesic activity

Analgesic activity was evaluated using the hot-plate method according to Woolfe [18]. Plate temperature was fixed at 52.5 ± 0.1 °C, and an arbitrary cut-off time of 45 s was adopted.

The analgesic potency of the compounds is reported as the ED_{50} (tables III, V). However, analgesic potency does not give indications as to the level of analgesia reached. To evaluate this parameter, the analgesic effect of the new products injected sc (icv in the case of the salts 2, 4 and 6) and their maximal nontoxic dose was compared to that of morphine as reference. This was injected at 8 mg/kg sc, a dose that does not alter animal behaviour.

Calculations were performed using the following formula: Analgesic efficacy of comp (X) expressed as % of that of morphine HCl (8 mg/kg sc) =

$\frac{\text{Maximum reaction time of } (X) - \text{pretest reaction of } (X)}{2} \times 100.$

Maximum react time morph – pretest react time morph

The maximal non-toxic dose is the highest dose of (X) which does not cause any visible change in animal behaviour, ie so that the researchers who were unaware of the treatment received by the animals were unable to distinguish between treated and non-treated mice.

Standard errors on the values expressed as percentage were not evaluated. Original data, however, were statistically processed by employing Dunnett's two-tailed test in order to verify the significance of the differences between the means shown by treated mice at the maximum reaction time and pre-test reaction time. Differences were considered to be statistically significant when $P \leq 0.05$. Percentage values were calculated only for those differences that were found to be statistically significant; in the other cases, the drugs were considered inactive.

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References

- 1 Gualtieri F, Romanelli MN, Scapecchi S et al (1991) Med Chem Res 1, 52-58
- 2 Ghelardini C, Giotti A, Gualtieri F et al (1992) Presynaptic auto- and heteroreceptors in the cholinergic regulation of pain. In: Trends in Receptor Research (Angeli P, Gulini U, Quaglia W, eds) Elsevier, Amsterdam, 95-114
- 3 Gualtieri F. Conti G. Dei S et al (1994) J Med Chem 37, 1704-1711
- 4 Ghelardini C, Gualtieri F, Romanelli MN et al (1997) Neuropharmacology 36, 281-294
- 5 Ghelardini C, Malmberg-Aiello P, Giotti A, Malcangio M, Bartolini A (1990) Br J Pharmacol 101, 49-54
- 6 Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A (1992) Br J Pharmacol 105, 77-82
- 7 Caulfield MP (1993) Pharmacol Ther 58, 319-379
- 8 Dei S, Bellucci C, Gualtieri F et al (1995) Il Farmaco 50, 303-309
- 9 Bartolini A, Ghelardini C, Gualtieri F et al (1989) Trends Pharmacol Sci suppl. 99
- 10 Gualtieri F, Ghelardini C, Giotti A, Malcangio M, Malmberg-Aiello P, Bartolini A (1989) Trends Pharmacol Sci suppl, 99

- 11 Chiarini A, Budriesi R, Bolognesi ML, Minarini A, Melchiorre C (1995) Br J Pharmacol 114, 1507–1517
- 12 Fodor G, Janzsó G, Ötvös L, Bánfi D (1960) Chem Ber 93, 2681-2685
- 13 Werner H (1918) J Am Chem Soc 40, 671-677
- 14 Fodor G, Rakoczi J, Csepreghy G (1961) Acta Chim Acad Sci Hung 28, 409-411
- 15 Caldwell HC, Finkelstein JA, Arbazov D, Pelikan C, Groves WG (1969) J Med Chem 12, 177-180
- 16 Dale AJ, Dull DL, Mosher HS (1969) J Org Chem 34, 2543-2549
- 17 Romanelli MN, Bartolini A, Bertucci C et al (1996) Chirality 8, 225-233
- 18 Woolfe G, Mcdonald A (1944) J Pharmacol Exp Ther 80, 300–307
- 19 Arunlakshana O, Schild HO (1959) Br J Pharmacol Chemother 14, 48–58
- 20 Tallarida RJ, Cowan A, Adler MW (1979) Life Sci 25, 637–654
- 21 Van Rossum JM (1963) Arch int Pharmacodyn 143, 299–330

- 22 Lefkowitz RJ, Cotecchia S, Samama P, Costa T (1993) Trends Pharmacol Sci 14, 303–307
- 23 Jakubik J, Bacalova L, El-Fakahany E, Tucek S (1995) FEBS Lett 377, 275-279
- 24 Kenakin T (1994) Trends Pharmacol Sci 15, 408-409
- 25 Kraiss G, Nádor K (1971) Tetrahedron Lett 57-58
- 26 Berthold H, Pfleger R, Schulz W (1967) Arzneim-Forsch 17, 719-726
- 27 Bryant III WM, Burlingame AL, House HO, Pitt CG, Tefertiller BAT (1966) J Org Chem 31, 3120–3127
- 28 Eltze M (1988) Eur J Pharmacol 151, 205-221
- 29 Eltze M, Gonne S, Riedel R, Schlotke B, Schudt C, Simon WA (1985) Eur J Pharmacol 112, 211–224
- 30 Eltze M, Figala V (1988) Eur J Pharmacol 158, 11-19
- 31 Dörje F, Friebe T, Tacke R, Mutschler E, Lambrecht G (1990) Naunyn Schmiedeberg's Arch Pharmacol 342, 284–289