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HL 752: A Potent and Long-Acting Antispasmodic Agent†

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Abstract—Ester analogues of methyl-2-(4-(2-piperidinoethoxy)benzoyl)-benzoate hydrochloride (pitofenone) (**2**) were prepared with an aim to find a more potent and metabolically stable antispasmodic compound. The compounds were evaluated for their in vitro and in vivo antispasmodic activity, and stability to in vitro enzymatic hydrolysis. Of the compounds synthesised, HL 752 (**21**) showed the most potent and long-lasting antispasmodic activity and was selected as the candidate for clinical development. © 1997 Elsevier Science Ltd.

Introduction

An abnormal spasm in gastrointestinal, ureteral, and uterine smooth muscles is the most frequent cause of abdominal discomfort. The causative factor for the spasm is not clearly understood. Generally, drugs like anticholinergics and nonspecific smooth muscle relaxants have been used clinically to relieve the symptoms. Atropine-like antispasmodics do not show selectivity towards a particular muscarinic receptor subtype and thereby they also show several side-effects. Their ready penetration of the central nervous system (CNS) can give rise to undesirable CNS effects. Synthesis of quaternary ammonium salts of the free bases serves as a useful technique to avoid or minimize these side-effects. However, absorption after oral administration is greatly reduced in spasmolytics containing the quaternary ammonium group, e.g. buscopan (**1**) (Chart 1).

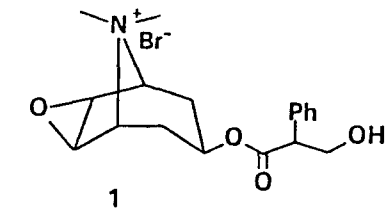
Pitofenone (**2**), possesses antispasmodic activity by virtue of anticholinergic and musculotropic activities in various smooth muscles. Even though the compound is well absorbed through the enteral route, pitofenone shows poor oral bioavailability due to a first-pass effect.[1] In an effort to find an antispasmodic compound with better metabolic stability than pitofenone, we synthesized a variety of ester analogues of pitofenone, in particular, sterically hindered esters, which might be expected to show delayed metabolism to the inactive acid **3**.

All derivatives were primarily evaluated for their antispasmodic activity in isolated guinea pig ileum using acetylcholine as a spasmogen. Compounds showing good IC_{50} values were further assessed for their in vivo activity with anesthetized dogs. In parallel, enzymatic hydrolysis studies were carried out to look for compounds with potential metabolic stability. This paper describes the synthesis and pharmacological evaluation of a variety of esters out of which the isopropyl ester, HL 752 (**21**) emerged as the compound for clinical development.

Chemistry

The two general approaches A and B which were adopted are summarized in Scheme 1. By route A, phthalein acid (2-(4-hydroxybenzoyl)benzoic acid) (**5**) could be converted to the target ester **4** in two steps: esterification with the appropriate alcohol **6** followed by alkylation of the phenolic ester **7** with piperidinoethyl chloride. Alternatively, by route B, the ester **4** could be obtained from acid **3** either by acid-catalyzed esterification (labeled (a) in Scheme 1), or by reaction of the alkali metal salt of acid **3** with the appropriate halide **8** (labeled (b) in Scheme 1).

Route A gave high yields with lower alcohols and could be readily applied to large-scale synthesis (Method A_1).



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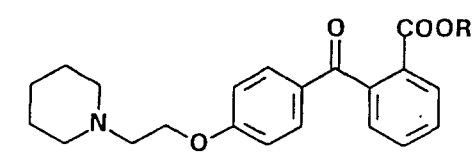

2 R = CH_3

3 R = H

Chart 1.

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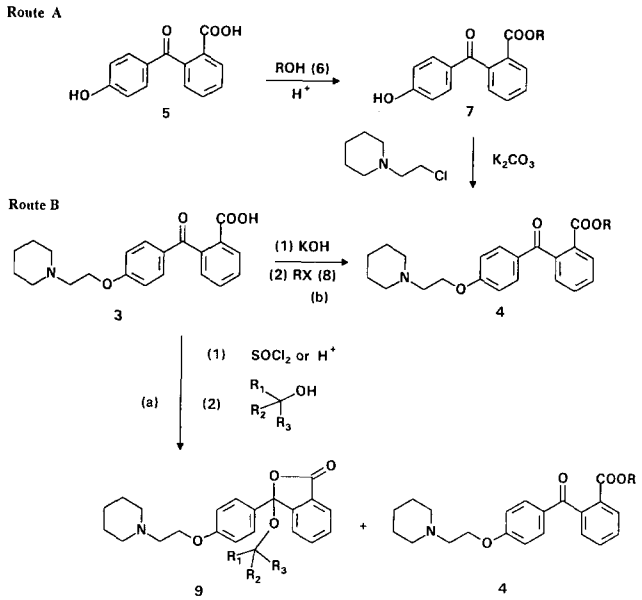


Scheme 1.

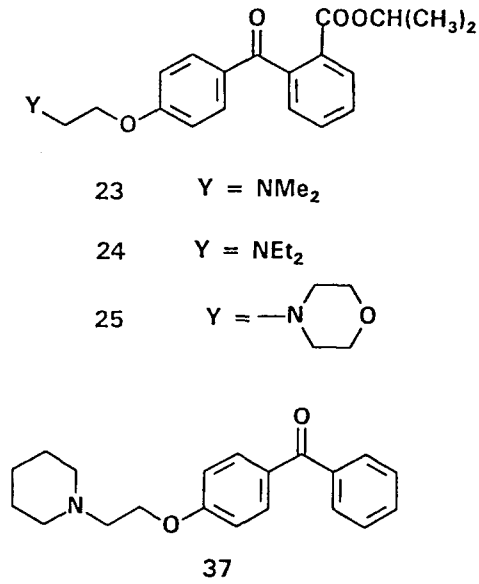


Chart 2.

For the synthesis of branched chain esters and esters of higher alcohols, the ordinarily slow esterification reaction could be accelerated by removal of water by drying agents like molecular sieves or anhydrous magnesium sulfate (Method A_2) or by azeotropic distillation. For synthesizing a series of ester derivatives, instead of the above two-step process, route B was expected to be more convenient. However, acid-catalyzed esterification of **3** with secondary alcohols led to low yields of **4**. A major by-product, and in some cases the only product, was the cyclized 'pseudo ester' **9** recognized by a characteristic IR band at 1780 cm^{-1} for a γ lactone, and in the case of the isopropyl derivative **9** (R_1 = H, R_2 = R_3 = CH_3), an upfield NMR shift of ca. 1 δ unit for proton R_1 compared to that expected for an isopropyl methine proton in ester **4** (R = $CH(CH_3)_2$).

The *tert*-butyl ester **26** was prepared by condensing acid **3** with *tert*-butanol in the presence of trifluoroacetic anhydride.[2] The cyclized compound **9** (R_1 = R_2 = R_3 = CH_3) was not detected in this reaction. Application of the TFA reaction to secondary alcohols, however, gave **9** as the major product.

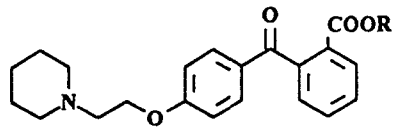
Reaction of acid **3** with alcohols in the presence of neutral condensing agents like DCC was difficult to carry out due to the insoluble nature of **3** in organic solvents or polar solvents like DMF or water. We then chose to alkylate the alkali metal salts of acid **3** which were either generated in situ or prepared and isolated prior to alkylation. A number of variations were tried.[3,4] Of these, the method that proved to be very simple and applicable to large-scale preparations was the solvent-free solid–liquid phase transfer catalyzed method,[4] in which the potassium salt of **3**, Aliquat 336, and the alkyl halide were allowed to react at or below the boiling point of the alkyl halide (Method B). When the potassium salt of **3** was treated with cyclopropyl bromide under the above conditions, the cyclopropyl ester was not formed. The only product isolated was the allyl ester **17.**

Since most of the free bases **4** were oils and only sparingly soluble in water, their pharmaceutical salts were prepared to get satisfactory microanalytical data and improve water solubility. The *tert*-butyl ester **26** was tested as the free base. Attempted salt formation of **26** led to ester hydrolysis in acidic medium. Four other compounds **23**, **24**, **25**, and **37** were prepared by standard methods for SAR studies (Chart 2).

Biochemistry

Pitofenone (**2**) is almost completely absorbed after oral administration.[1] The low serum levels and the inverse profile after iv administration have been attributed to a possible hepatic and/or pulmonary first-pass effect.[1] One of the plausible explanations for the short duration of oral activity of pitofenone may be its rapid metabolism to the inactive pitofenone acid **3**. Accordingly, we examined the in vitro hydrolysis of pitofenone in the presence of proteolytic enzymes (such as trypsin, chymotrypsin, and pepsin) and tissue homogenates of lung, liver, intestine, kidney, and heart. Under standard assay conditions, the compound was hydrolysed to pitofenone acid **3**, predominantly by liver and to some extent by lung homogenates. Other tissue extracts and proteolytic enzymes (as mentioned above, data not shown) were ineffective. Further, an in vivo radiotracer study has identified liver as the principal site for the metabolism of pitofenone.[1] These results, taken together with the observed hepatic first-pass effect, clearly implicate a liver esterase for the hydrolytic inactivation of pitofenone. Indeed, purified commercial porcine liver esterase rapidly hydrolysed this compound (Table 1) to the acid **3**. We therefore reasoned that the liver esterase is a good in vitro enzyme screen to test for

Table 1. Physical and biological data for analogues of methyl-2-(4-(2-piperidinoethoxy)benzoyl)-benzoate (**2**)



Compd	R	Method[a]	Yield[b]	Molecular formula	Mp[c] (°C)	IC_{50}[d] (μM)	Hydrolysis catalyzed by esterase[e] 1 h	2 h	4 h
3	H					>28			
2	CH_3			$C_{22}H_{25}NO_4 \cdot HCl$		0.74	++	+++	+++
10[f]	CH_3		82	$C_{22}H_{25}NO_4 \cdot CH_3I$	154–156	6.30	+	++	+++
11	C_2H_5	A_1	49, 83	$C_{23}H_{27}NO_4 \cdot HCl$	180–181	0.03	++	+++	+++
12	$(CH_2)_2CH_3$	A_1	80, 72	$C_{24}H_{29}NO_4 \cdot HCl \cdot 0.25H_2O$	100–102	0.18	+++	+++	+++
13	$(CH_2)_3CH_3$	B	80	$C_{25}H_{31}NO_4 \cdot HCl$	129–131	0.28	+++	+++	+++
14	$(CH_2)_4CH_3$	A_1	95, 51	$C_{26}H_{33}NO_4 \cdot HCl$	104–105	0.20	+++	+++	+++
15	$CH_2CH(CH_3)_2$	A_1	81, 47	$C_{25}H_{31}NO_4 \cdot HCl \cdot 0.25H_2O$	85–86	0.48	++	+++	+++
16	$CH_2C(CH_3)_3$	B	22	$C_{26}H_{33}NO_4 \cdot HCl \cdot H_2O$	83–86	3.30	–	–	–
17	$CH_2CH=CH_2$	B	4	$C_{24}H_{27}NO_4 \cdot HCl$	144–146	0.54	++	+++	+++
18	$CH_2CH=C(CH_3)_2$	B	11	$C_{26}H_{31}NO_4 \cdot HCl \cdot H_2O$	94–97	2.10	+++	+++	+++
19	CH_2Ph	B	36	$C_{28}H_{29}NO_4 \cdot HCl$	155–158	0.03	++	+++	+++
20	$CH_2C_6H_4(4COOMe)$	A_2	3, 54	$C_{30}H_{31}NO_6 \cdot HCl$	126–128	1.86	+++	+++	+++
21	$CH(CH_3)_2$	A	85, 90	$C_{24}H_{29}NO_4 \cdot HCl$	97–99	0.02	–	–	–
(HL 752)		B	88						
22[f,g]	$CH(CH_3)_2$		45	$C_{24}H_{29}NO_4 \cdot CH_3I$, $0.75H_2O$	109–110	0.03			
23[f,h]			75[i]	$C_{21}H_{25}NO_4 \cdot HCl$, $0.75H_2O$	77–80	0.67	–	–	–
24[f,h]			86[i]	$C_{23}H_{29}NO_4 \cdot HCl$, $0.5H_2O$	99–101	1.27	–	–	–
25[f,h]			91[i]	$C_{23}H_{27}NO_5 \cdot HCl$, H_2O	142	1.77	–	–	–
26[f,g]	$C(CH_3)_3$		18	$C_{25}H_{30}NO_4$	Oil	1.03			
27	$CH(CH_3)C_2H_5$	B	61	$C_{25}H_{31}NO_4 \cdot HCl$	125–127	1.17	–	–	–
28	$CHEt_2$	B	82	$C_{26}H_{33}NO_4 \cdot HCl$	129–131	2.96	–	–	–
29	$CH(CH_3)CH_2Ph$	B	58	$C_{30}H_{33}NO_4 \cdot HCl$, H_2O	126-129	3.81	–	+	+
30	$CH(CH_3)CH(CH_3)_2$	A_2	59, 70	$C_{26}H_{33}NO_4 \cdot HCl$	127–129	0.10	–	–	–
31	$CH(CH_3)tBu$	A_2	76, 73	$C_{27}H_{35}NO_4 \cdot HCl$	138–140	16.00	–	–	–
32	Cyclopentyl	B	88	$C_{26}H_{31}NO_4 \cdot HCl$	139–140	1.11	–	–	–
33[g]	Cyclohexyl	B	25	$C_{27}H_{33}NO_4 \cdot HCl$, $1.5H_2O$	120	2.30			
34	$CH(CH_3)COCH_3$	B	68	$C_{25}H_{29}NO_5 \cdot HCl \cdot 1.5H_2O$	107–110	3.70	–	–	++
35	$CH(CH_3)CH=CH_2$	B	61	$C_{25}H_{29}NO_4 \cdot HCl$	112–114	0.19	+	+	++
36	$CH(CH_3)CH_2OCH_3$	A_2	32, 86	$C_{25}H_{31}NO_5 \cdot HCl$	88–90	10.18	–	–	+
37[f,h]			97	$C_{20}H_{23}NO_2 \cdot HCl$	173–174	10.99			
Buscopan						0.18			

[a]Letters refer to the method of preparation described in the Experimental section.
[b]For Methods A_1 and A_2, the first yield refers to the conversion of compound **5** to **7**, and the second yield refers to the conversion of compound **7** to **4** (Scheme 1). For Method B, the yield refers to the conversion of compound **3** to **4**. The yields are not optimized.
[c]mp of the salt.
[d]IC_{50} values were determined graphically from dose–response curves obtained by measuring antispasmodic activity at different concentrations of the compounds in duplicate or triplicate.
[e]Ester hydrolysis was monitored semiquantitatively, by the appearance of acid **3**, at the end of the incubation with the esterase (see Experimental section). The extent of reaction was classified as: 90% (+++), 40–60% (++), <20% (+) or no (–) hydrolysis of the initial ester.
[f]For preparation see Experimental section.
[g]The extent of hydrolysis could not be determined due to limited solubility.
[h]See text for structure.
[i]Yield from **7** (R = isopropyl).

potential metabolic stability of pitofenone derivatives. Accordingly the hydrolytic reaction of the pitofenone analogues described above was studied in the presence of porcine liver esterase and the results are shown in Table 1.

Results and Discussion

In the search for a potent antispasmodic compound with good metabolic stability, modifications in the parent molecule **2** might be envisaged at various sites:

Table 2. Antispasmodic activity in anesthetised dog

Compd	ID_{50}[a] (mg kg^{-1}) iv
2	0.14
11	0.03
12	0.08
19	1.08
21	0.01 (6)[b]
30	0.77
35	0.30

[a]ID_{50} values were determined graphically from dose–response curves obtained by measuring antispasmodic activity at different concentrations of compounds in triplicate.
[b]Number of observations exceeding three are mentioned in parentheses.

the amino group, the aminoalkoxy chain, the aromatic rings, the benzophenone carbonyl, and the ester group. The observed first-pass effect[1] with pitofenone (**2**) and the postulation of the inactive pitofenone acid **3** as its possible metabolite, suggest a role for the liver carboxylesterase (EC 3.1.1.1) in the metabolism of pitofenone (**2**). Mammalian liver esterases are often implicated in drug detoxification. Most likely, the catalytic mechanism of such enzymes involves a serine-OH at the active site.[5] The stereochemical access to the carbonyl group of the ester **2** by this nucleophile could determine substrate hydrolytic rates. Therefore, our initial efforts were directed towards altering the ester functionality in order to improve its stability while retaining antispasmodic activity. The effect of chain length and steric bulk in the ester unit on antispasmodic activity as well as on hydrolytic rates was studied. Some branched chain esters were synthesized anticipating that this may reduce ester hydrolysis to the inactive acid **3**. Allyl and substituted allyl derivatives as well as benzyl and substituted benzyl compounds were included in the study; the effect of cycloalkyl esters was also investigated.

Although the number of compounds in the series is too small for detailed structure–activity relationships to be derived, several trends emerge on examination of the IC_{50} data in Table 1, and a few general comments can be made. An ester with a two carbon unit shows better antispasmodic activity than the methyl ester. Substitution of the α-hydrogen with a methyl group improves antispasmodic activity in general, as can be seen with the pairs of compounds **11** and **21**, **15** and **30**, and **17** and **35**. The active site seems sensitive to steric crowding. The following groups are not tolerated and lead to reduced activity: an ethyl branch (compounds **27** and **28**), a *tert*-butyl group (compounds **16**, **26**, and **31**), a dimethyl group on a double bond (**18**) and a cycloalkyl moiety (compounds **32** and **33**). Preliminary studies at the amino site in the molecule suggest that the piperidino group appears essential for antispasmodic activity.

On the basis of in vitro data, compounds **11**, **12**, **19**, **21**, **30**, and **35** which showed potent antispasmodic activity (Table 1), were further evaluated for in vivo antispasmodic activity in the anesthetized dog. All these compounds, administered intravenously, showed dose-related antispasmodic activity against carbachol-induced intestinal contractions (Table 2). Of these, compounds **21** and **11** showed far superior activity compared to compound **2**, with **21** being the better of the two. In addition, the duration of antispasmodic activity for compound **21** was significantly higher than that for the ethyl ester **11**. This observation was further ascertained in another set of experiments conducted in the anesthetized dog, in which compounds **21** and **11** were administered intraduodenally. In this experiment, compound **21** (ID_{50} = 0.37 mg kg^{-1}) not only showed more potent antispasmodic activity than **11** (ID_{50} > 1 mg kg^{-1}) but also a more prolonged duration of activity (Table 3). On the basis of in vitro and in vivo pharmacological studies, the isopropyl ester appeared to have a better profile than the ethyl ester.

To further evaluate the potential metabolic stability of the various ester analogues of **2**, enzymatic studies were conducted in parallel (Table 1). From esterase hydrolysis data in Table 1 it is clear that esters of secondary alcohols and those with *tert*-butyl groups are, in general, resistant to hydrolysis catalyzed by esterase. However, since many of these esters with bulky groups did not show good antispasmodic activity, they were not selected for further pharmacological studies. Of the two compounds of our interest, the ethyl ester **11** was rapidly hydrolysed by liver esterase while the isopropyl ester **21** was remarkably stable. Compound **21** also proved to be resistant to hydrolysis in the presence of other proteolytic enzymes and tissue homogenates.

Table 3. Antispasmodic activity of compounds **21** and **11** administered id in anesthetized dog[a]

Compd	Dose (mg kg^{-1} id)	*n*	Percent change in intestinal contraction	Duration of action (min)	ID_{50} (mg kg^{-1})
11	0.1	3	5±5	3±3	≈1.0
	0.3	4	19±9	10±4	
	1.0	4	47±18	27±9	
21	0.1	5	36±13	18±10	0.37
	0.3	6	41±12	54±24	
	1.0	5	74±6	113±33	

[a]Pitofenone (compound **2**) showed no effect up to a dose of 10 mg kg^{-1}.

As a result of studies to determine the selectivity of compound **21** (HL 752) towards the M_1, M_2 and M_3 muscarinic receptor subtypes by radioligand binding assay, HL 752 was found to be a pure competitive muscarinic receptor antagonist with selectivity towards the M_3 receptor subtype.[6]

Compound **21** administered iv at doses up to 3 mg kg^{-1} in the anesthetized dog did not show any significant change in cardiovascular parameters like systolic and diastolic blood pressure, heart rate, and left ventricular dP/dt max. In sub-acute toxicity studies in the rat, administration of 100 mg kg^{-1} po of compound **21** daily for 21 days did not result in adverse effects on biochemical and hematological parameters.

Finally, on the basis of all the above observations, compound **21** (HL 752) was adjudged the best compound of the series and was selected as a candidate for clinical development.

Experimental

Chemistry

Melting points were determined on a Thomas–Hoover apparatus and are uncorrected. ^1H NMR spectra were recorded on a JEOL-90-Q or a Varian T-60 spectrometer. Chemical shifts are reported in δ units downfield from the internal standard tetramethylsilane. The coupling constants (J) are in hertz. IR spectra were recorded on a Perkin–Elmer 157 spectrometer and are reported in reciprocal centimeters (cm^{-1}). For the compounds described, analyses of C, H, N, Cl, and I were within ±0.4% of the theoretical values.

Acid **3** was prepared by hydrolysis of pitofenone[7] with NaOH in methanol–water at 70–75 °C, followed by cooling, acidification to pH 7.8 with concd HCl and filtration of the resulting solid. The potassium salt was prepared by the reaction of acid **3** and 1 equiv KOH in dry methanol followed by removal of solvent and thorough drying under vacuum at 60 °C. The *tert*-butyl ester (**26**) was prepared as described in ref 2. The methiodides **10** and **22** were prepared by treating free base **4** with methyl iodide in methanol. Compounds **23**, **24**, and **25** were prepared as described in Method A_2 below using dimethylaminoethyl chloride, diethylaminoethyl chloride and morpholinoethyl chloride respectively as alkylating agents instead of piperidinoethyl chloride. Compound **37** was prepared by the alkylation of 4-hydroxybenzophenone with piperidinoethyl chloride.

Method A_1

General method for the synthesis of compound 4. A mixture of acid **5** (0.015 mol), concd H_2SO_4 (1.5 mL), and alcohol **6** (60 mL) was refluxed. On completion of reaction as determined by thin-layer chromatography (TLC), the alcohol was distilled off in vacuo. The residue was partitioned between ethyl acetate and water. The organic phase was washed with water, followed by satd $NaHCO_3$ solution, dried (Na_2SO_4) and concentrated to give the required intermediate **7**.

The phenolic ester **7** was alkylated with piperidinoethyl chloride as described in Method A_2 to give the required product **4.**

Method A_2

(3-Methylbut-2-yl)-2-(4-(2-piperidinoethoxy)-benzoyl)-benzoate (30). A mixture of acid **5** (15 g, 0.062 mol), 3-methyl-2-butanol (100 mL), concd H_2SO_4 (3 mL) and toluene (100 mL) was placed in a flask fitted with a Soxhlet extractor charged with 3 Å molecular sieves (25 g) or a filter paper packet containing anhydrous Na_2SO_4 (50 g). The reaction mixture was refluxed for 4 h after which it was poured on crushed ice containing satd $NaHCO_3$ (60 mL). The mixture was extracted with EtOAc (2 × 500 mL). The organic layer was washed with water, dried over Na_2SO_4 and concentrated to give 11.5 g (59%) of intermediate **7** [R = $CH(CH_3)CH(CH_3)_2$] as a viscous oil.

A mixture of the above ester **7** (10 g, 0.032 mol), ethyl acetate (80 mL, containing 4.6% water), piperidinoethyl chloride hydrochloride (7.58 g, 0.042 mol), and anhydrous K_2CO_3 (11.5 g, 0.083 mol) was refluxed for 4 h. After cooling, the solid residue was filtered off. The filtrate was washed with water (2 × 300 mL), dried, and concentrated in vacuo to give 10.2 g of a dark oil which was purified by filtration over neutral alumina. Elution with 10% EtOAc–petroleum ether and concentration gave 9.5 g (70%) of the title compound as a viscous oil. IR (neat): 3060, 2940, 1715, 1665, 1600 cm^{-1}; NMR ($CDCl_3$): 7.92–8.02 (m, 1H), 7.19–7.40 (m, 2H), 7.40–7.75 (m, 3H), 6.72–6.92 (m, 2H), 4.74 (dq, 1H, 6.5 Hz), 4.08 (t, 2H, 6.5 Hz), 2.72 (t, 2H, 6.5 Hz), 2.30–2.56 (m, 4H), 1.0–1.78 (m, 7H), 0.95 (d, 3H, 6.5 Hz), 0.76 (d, 6H, 7.2 Hz).

Method B

Isopropyl-2-[4-(2-piperidinoethoxy)-benzoyl]benzoate (21). To a mixture of 2-bromopropane (29.8 g, 0.24 mol) and Aliquat 336 (10.7 g, 0.026 mol) was added dry powdered potassium salt of **3** (103.2 g, 0.26 mol). The flask was fitted with a $CaCl_2$ guard tube and shaken for 15 min. It was then fitted with a reflux condenser and the reaction mixture heated at 60 °C (bath temperature) for 25 h. The product was triturated with 25% EtOAc–petroleum ether and filtered over a bed of grade I neutral alumina followed by elution with 25% EtOAc–petroleum ether. The combined filtrate was concentrated to give 84 g (88%) of the title compound as a colorless viscous oil which could be crystallized from *n*-pentane at ca. −6 °C to give white crystals; mp 34–35 °C. IR (neat): 3000, 2950, 1720, 1670, 1600 cm^{-1}; NMR

($CDCl_3$): 7.92–8.02 (m, 1H), 7.19–7.7 (m, 5H), 6.72–6.92 (m, 2H), 4.96 (h, 1H, 5.8 Hz), 4.12 (t, 2H, 6.5 Hz), 2.78 (t, 2H, 6.5 Hz), 2.40–2.60 (m, 4H), 1.40–1.75 (m, 6H), 1.06 (d, 6H, 5.8 Hz).

Typical procedure for the preparation of hydrochloride salts: Isopropyl-2-[4-(2-piperidinoethoxy)benzoyl]-benzoate hydrochloride (21 HCl). A total of 6.5 g (0.016 mol) of compound **21** was dissolved in 35 mL dry dichloromethane. Ethereal HCl was added dropwise until the pH reached 3. The solvent was distilled off in vacuo and the residue triturated with ether to get a solid. Crystallization from EtOAc gave 4.87 g (68%) of the hydrochloride salt as colorless crystals, mp 97–99 °C.

Biochemistry

Enzymatic hydrolysis of pitofenone and other esters. Unless otherwise stated, the standard esterase reaction mixture (1 mL) contained 40 mM Tris–HCl, pH 8.2, porcine liver esterase (0.1 unit) and the test compound (0.1–1.0 mM range). After incubation at 37 °C, the reaction was terminated by placing the assay tubes in a boiling water bath for 2 min. Appropriate blanks without enzyme were included in each experiment to account for non-enzymatic hydrolysis. The extent of hydrolysis was monitored by TLC. A 20 μL aliquot of the reaction mixture was spotted on TLC plates (Merck, Kieselgel 60 F 254) and the chromatograms (solvent–chloroform:methanol 4:1) were examined under UV light (254 nm). The R_f values for pitofenone and its metabolite, pitofenone acid, were 0.7 and 0.2, respectively. The extent of reaction was scored by comparing the parent ester spot with the product acid spot. The minimum visual detectable limit of pitofenone acid **3** was 1 nmol.

The possible hydrolysis of pitofenone and its isopropyl derivative by various tissue homogenates was followed essentially as described above except that the reaction was terminated by the addition of 0.25 mL of 1.0 N acetic acid and then the protein precipitate was removed by centrifugation prior to TLC. Tissue extracts were prepared as mentioned[8] and the protein was determined by biuret method.[9]

Pharmacological methods

Antispasmodic activity: isolated guinea pig ileum.[10] Antispasmodic activity was assessed in isolated guinea pig ileum using acetylcholine as a spasmogen. The ileum of freshly sacrificed guinea pig of either sex, weight range 200–300 g, was removed and suspended in an organ bath containing Tyrode solution. The bath temperature was maintained at 37 °C and aerated with compressed air. Basal tension of 0.5–1 g was given. Tension changes in the tissues were monitored using Hugo Sachs force displacement transducer (K-30) amplifier and recorded on a four-channel potentiometric recorder. After 30 min of equilibration period, dose–response curve for acetylcholine was constructed to select a submaximal dose. After taking the control responses with submaximal dose of acetylcholine, tissue was treated with test compounds at different concentrations. A contact time of 1 min was given to each test compound. The antispasmodic activity was assessed as a percent reduction over the initial value. All the compounds were dissolved in distilled water (1 mg mL^{-1}). The IC_{50} values were calculated from the dose–response curve. Buscopan was used as a standard compound.

Antispasmodic activity: Anesthetized dog.[11] Male mongrel dogs of weight range 10–15 kg were anesthetized with pentobarbitone sodium (35 mg kg^{-1}, iv). Slow iv infusion of pentobarbitone (5 mg kg^{-1} h^{-1}) was given as maintenance anesthesia. The endotracheal tube was inserted to facilitate spontaneous breathing. Both femoral artery and vein were cannulated for recording blood pressure and administering compound, respectively. A part of small intestine distal to the duodenum was isolated and a saline-filled balloon catheter was inserted and the opening was sutured. A fine polyethylene cannula was then inserted into the mesenteric artery which supplies the area of intestine containing the balloon for injection of carbachol. A Statham pressure transducer was then attached to the balloon catheter for recording both circular and longitudinal muscle contractions. All parameters were recorded on a four-channel Nihon–Kohden recorder. The control intestinal contractions were recorded after administering a standard dose of carbachol (1 μg, ia).

Compounds were dissolved in distilled water (concentration 1–10 mg mL^{-1}). Each dose of test compound was administered iv or id in a volume of 0.1 mL kg^{-1}. According to in vitro antispasmodic activity, doses were selected between 0.01 and 10 mg kg^{-1}. Three to five doses were selected in order to establish the dose–response relationship. A 10–30 min interval was given in between the doses. In each experiment, the activity of one compound was assessed. The antispasmodic activity was determined by estimating the reduction in contraction seen after the administration of test compound as compared to the initial values. The duration of antispasmodic activity was assessed by challenging the preparation with carbachol at different time intervals until the contraction returned to control value. ID_{50} value was calculated from the dose–response curve.

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