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Synthesis and pharmacological evaluation of a set of *N*-[2-(alkylamino)ethyl]benzotriazol-*X*-yl isobutyramides acting as local anesthetics

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Summary — A set of *N*-[2-(alkylamino)ethyl]benzotriazol-*X*-yl isobutyramides have been synthesized and assayed in vivo for their local anesthetic activity and acute toxicity. Some of them showed a more favorable pharmacological profile than that of the reference drug lidocaine. QSAR studies have delineated the influence of overall lipophilicity on surface and infiltration anesthetic activities.

benzotriazole / local anesthetic / acute toxicity / QSAR / lipophilicity

Introduction

We have recently reported the synthesis, the pharmacological evaluation and a QSAR study for a series of *N*-[2-(alkylamino)ethyl]benzotriazol-*X*-yl acetamides (**1a–f** and **2a–f** in chart 1) acting as local anesthetics [1]. The compounds were designed so as to vary independently the hydrophobicity and the bulkiness of the amine moieties. Among the tested benzotriazoles, some of them (**2b–d**) showed surface (rabbit cornea assay) and infiltration (mouse tail assay) anesthetic activities comparable with or even higher than those exhibited by the reference drug lidocaine.

A QSAR analysis conducted in this data set revealed that both surface and infiltration anesthesia were enhanced by increasing overall CLOGP [2] values. These findings suggested that more effective anesthetic benzotriazoles could be obtained by further increasing the hydrophobicity of the molecules. Thus, we decided to insert two methyl groups on the carbon atom attached to the benzotriazole ring of **1a–f** and **2a–f**. Such a structural modification was expected to exert a favorable effect on anesthetic activity owing to the 0.62 units increase in the CLOGPs of the isobutyramides **3a–f** and **4a–f** (chart 1) compared with their corresponding **1a–f** and **2a–f** homologs. It was also hoped that the two methyl groups could further improve the pharmacological profile of the target compounds through an intramolecular conformational effect consisting of a loss of conformational freedom about the N–C(Me$_2$)–C(=O) bonds. Although the influence of this effect was not predictable on the basis of our previous study [1], we nevertheless thought it worthy of specific investigation.

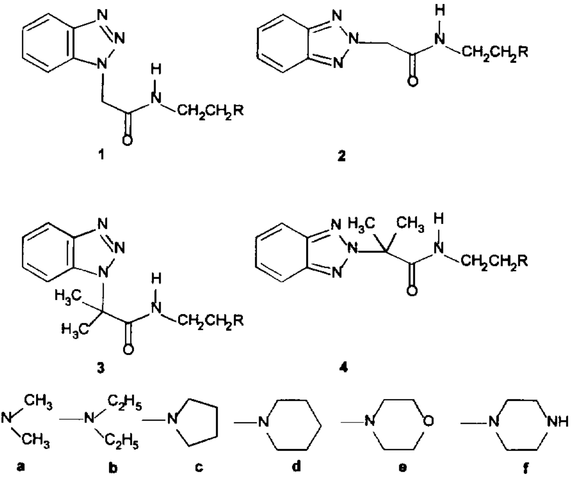

Chart 1.

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In the present paper we describe the synthesis, the pharmacological testing and QSAR studies for *N*-[2-(alkylamino)ethyl]benzotriazol-*X*-yl isobutyramides **3a–f** and **4a–f**.

Chemistry

All target compounds *N*-[2-(alkylamino)ethyl]benzotriazol-*X*-yl isobutyramides **3** and **4** were satisfactorily obtained as hydrochlorides by condensation of benzotriazolylisobutyric acids **7** or **8**, using DCC as a condensing reagent, with the appropriate amine in DMF followed by treatment with hydrochloride acid (scheme 1).

The starting compounds **5** and **6** were prepared by reaction of benzotriazole with ethyl 2-bromoisobutyrate in absolute ethanol and sodium ethoxide according to a published method [3]. During the course of the addition reaction it was verified that 1- and 2-substituted isomers **5** and **6** formed in a considerable amount, generally with an overall yield of 60%. Separation of the benzotriazole derivatives obtained **5** and **6** was performed by chromatography on a silica-gel column using diethyl ether/*n*-hexane 7:3 v/v as eluent. These derivatives were successively converted into the corresponding carboxylic acids by treatment with 10% NaOH and subsequent treatment with 1 N HCl.

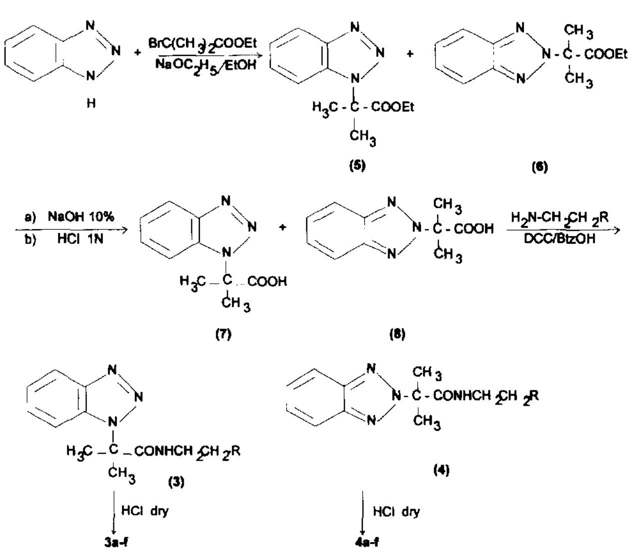
All the final compounds gave satisfactory elemental analyses and their UV and ^{1}H-NMR spectra were consistent with the proposed structures.

The ^{1}H-NMR spectra differentiated clearly between 1- and 2-benzotriazole isomers. Proton signals of the 2-benzotriazolyl fragments formed a diagnostic, symmetrical pattern in the spectrum [4]. The side chain [$aC(CH_3)_2CONH\text{-}bCH_2\text{-}cCH_2\text{-}N(CH_3)_2$] of compound **4a**, taken as an example, is characterized by the following chemical shifts: δ 3.60 (2H, t, J = 7.5 Hz, bCH_2), 3.39 (2H, t, J = 7.5 Hz, cCH_2), 2.98 (6H, s, $aC(CH_3)_2$) and 2.10 (6H, s, $N(CH_3)_2$). The chemical shifts of the side chain of the corresponding 1-isomer (**3a**) were the following: δ 3.58 (2H, t, J = 7.5 Hz, bCH_2), 3.28 (2H, t, J = 7.5 Hz, cCH_2), 2.95 (6H, s, $aC(CH_3)_2$) and 2.10 (6H, s, $N(CH_3)_2$).

Similar ^{1}H-NMR data were found in all the other benzotriazole derivatives. Physicochemical data of all the final compounds **3a–f** and **4a–f** are summarized in table I.

Pharmacology

The local anesthetic effect of all compounds was evaluated in vivo by corneal anesthesia in rabbit and mouse tail anesthesia (table II). From the results of these tests, the most active compounds were selected for further studies on the rat sciatic nerve block anesthesia, ip acute toxicity and therapeutic index. These results are reported in table III. The activities of the analyzed compounds were compared with those of the reference drug lidocaine.



Scheme 1.

Results and discussion

Table II summarizes the results of the surface and infiltration anesthesia assays performed on benzotriazoles **3a–f** and **4a–f**. Table IV lists the same activity values on a logarithmic scale for all the benzotriazoles synthesized and tested to date (compounds **1–4**). Three of the recently prepared isobutyramides, namely **4b**, **4c** and **4d**, exhibit anesthetic activities higher than those displayed by lidocaine in both pharmacological assays. However, most of the isobutyramides were found less active in the surface anesthesia test than predicted according to the eq [1], previously derived from acetamides **1a–f** and **2a–f** [1].

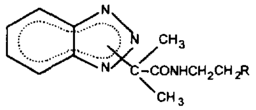
$$\log \text{AUC} = 1.22\ (\pm 0.31)\ \text{CLOGP} - 0.97\ (\pm 0.51) \qquad [1]$$

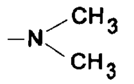
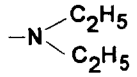
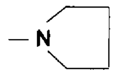
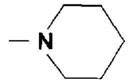
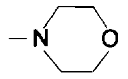
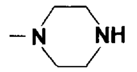
$$n = 12,\ r_f^2 = 0.882,\ r_{cv}^2 = 0.854,\ s = 0.281,\ F_{1,10} = 74.54$$

In the above equation n is the number of data points, r_f^2 and r_{cv}^2 are the squared correlation coefficients of the calibrated and, respectively, cross-validated models [5], s is the standard error of the calibration model, the numbers in parentheses are the 95% fiducial limits; the F-value is calculated over the cross-validated residuals.

It is likely that the insertion of the two methyl groups exerts a detrimental effect on anesthetic acti-

Table I. Physicochemical properties of benzotriazole derivatives **3a–f** and **4a–f**.



R	*Formula*[a]	*MW*	*1-Substituted benzotriazoles 3a–f* Compound	Yield (%)	Mp (°C)	Recrystallization solvent[b]	*2-Substituted benzotriazoles 4a–f* Compound	Yield (%)	Mp (°C)	Recrystallization solvent[b]
	$C_{14}H_{21}N_5O{\cdot}HCl$	311.81	**3a**	75	171–173	a + b	**4a**	75	200–201	a + b
	$C_{16}H_{25}N_5O{\cdot}HCl$	339.86	**3b**	55	144–146	a + b	**4b**	75	141–142	a + b
	$C_{16}H_{23}N_5O{\cdot}HCl$	337.85	**3c**	73	189–190	a + b	**4c**	55	194–195	a + b
	$C_{17}H_{25}N_5O{\cdot}HCl$	351.88	**3d**	52	144–145	a + b	**4d**	85	210–212	a + b
	$C_{16}H_{23}N_5O_2{\cdot}HCl$	353.85	**3e**	50	212–213	c + b	**4e**	83	181–183	c + b
	$C_{16}H_{24}N_6O{\cdot}2HCl$	389.33	**3f**	40	211–212	c + b	**4f**	50	252–254	c + b

[a]Satisfactory microanalyses obtained: C, H, N values are within ±0.4% of the theoretical ones; [b]recrystallization solvents: a) ethyl alcohol; b) diethyl ether; c) methyl alcohol.

vity which would compensate the higher lipophilicity of the isobutyramide derivatives (0.62 more lipophilic than their corresponding acetamide homologs).

Eq [2] was obtained from the log AUC and CLOGP data of compounds **3a–f** and **4a–f**.

$$\log \mathrm{AUC} = 1.32\ (\pm 0.54)\ \mathrm{CLOGP} - 2.0\ (\pm 1.2) \qquad [2]$$
$$n = 12,\ r_f^2 = 0.750,\ r_{cv}^2 = 0.651,\ s = 0.480,\ F_{1,10} = 18.69$$

Observed and calculated log AUC values from eqs [1] and [2] are listed in table IV. A plot of log AUC versus CLOGP is given in figure 1.

The similarity of the coefficients of CLOGP in eqs [1] and [2] supports a common mechanism of action for the acetamide and isobutyramide derivatives. The difference between the two intercepts (about 1 log AUC unit) quantifies the hypothesized unfavorable steric effect of the geminal methyl groups. By inspecting the diagram in figure 1, we can safely exclude the existence of a parabolic or bilinear relationship between log AUC and CLOGP.

Concerning the infiltration anesthesia, in addition to the previously reported eq [3] [1] for acetamides **1a–f** and **2a–f**, we also derived eq [4] from the data relative to isobutyramides **3a–f** and **4a–f**.

$$\log 1/\mathrm{C}_{50} = 0.60\ (\pm 0.26)\ \mathrm{CLOGP} + 0.42\ (\pm 0.42) \qquad [3]$$
$$n = 12,\ r_f^2 = 0.728,\ r_{cv}^2 = 0.627,\ s = 0.231,\ F_{1,10} = 26.69$$

$$\log 1/\mathrm{IC}_{50} = 0.53\ (\pm 0.34)\ \mathrm{CLOGP} + 0.34\ (\pm 0.76) \qquad [4]$$
$$n = 12,\ r_f^2 = 0.546,\ r_{cv}^2 = 0.285,\ s = 0.305,\ F_{1,10} = 3.98$$

The significance level of eq [4], according to the F-test on the cross-validated residuals, is below 95%. By omitting the poorest predicted data point (compound **3d**), we derived eq [5] with a significance level above 99.25% and an r_{cv}^2 value about two times higher.

$$\log 1/\mathrm{IC}_{50} = 0.65\ (\pm 0.30)\ \mathrm{CLOGP} + 0.13\ (\pm 0.65) \qquad [5]$$
$$n = 11,\ r_f^2 = 0.729,\ r_{cv}^2 = 0.594,\ s = 0.246,\ F_{1,9} = 13.19$$

Observed and calculated $1/\mathrm{IC}_{50}$ values from eqs [3] and [5] are listed in table IV. A scatter diagram of log $1/\mathrm{IC}_{50}$ versus CLOGP is reported in figure 2.

Table II. Rabbit corneal and mouse tail anesthetic activities.

Compound	Corneal anesthesia[a]	Mouse tail anesthesia[b]
3a	1.0 ± 1.5	5.0 (± 0.34) x 10^{-2}
3b	2.7 ± 2.0	3.6 (± 0.34) x 10^{-2}
3c	18.4 ± 3.8	5.2 (± 0.36) x 10^{-2}
3d	13.0 ± 4.0	5.0 (± 0.38) x 10^{-2}
3e	1.1 ± 1.0	1.2 (± 0.48) x 10^{-1}
3f	1.2 ± 2.0	4.5 (± 0.32) x 10^{-2}
4a	1.5 ± 1.3	4.1 (± 0.33) x 10^{-2}
4b	177 ± 15.0	9.5 (± 0.30) x 10^{-3}
4c	173 ± 13.8	9.5 (± 0.33) x 10^{-3}
4d	114 ± 10.0	4.5 (± 0.31) x 10^{-3}
4e	2.0 ± 0.9	3.4 (± 0.35) x 10^{-2}
4f	1.0 ± 1.2	1.1 (± 0.45) x 10^{-1}
Lidocaine•HCl[c]	100	1.6 (± 0.43) x 10^{-2}

[a] All compounds were in aqueous solution at 2% concentration. The values, expressed as % of the anesthetic activity of lidocaine (= 100), are means ± SE of three determinations; [b] IC_{50} values expressed as mol/L; [c] lidocaine hydrochloride was used for comparison.

Table III. Duration of local anesthetic activity (rat sciatic nerve), acute toxicity in mouse and therapeutic index for a subset of benzotriazole derivatives.

Compound	Duration[a] (min)	LD_{50}[b] (mg/kg)	LD_{50}/IC_{50}[c]
4b	133	122	7.6
4c	123	90	5.6
4d	100	75	9.4
2b[d]	103	160	10.3
2c[d]	90	150	7.8
2d[d]	113	70	4.4
Lidocaine•HCl[d]	110	110	5.1

[a] In vivo duration of local anesthetic activity in rat sciatic nerve block (each rat received 0.2 mL of 2% anesthetic solution); [b] acute toxicity in male mouse (ip administration); [c] IC_{50} values correspond to the mouse tail test (infiltration anesthesia) and are expressed in mg/kg; [d] data taken from reference [1].

Table IV. Data used to derive eqs [1–5].

Compound	log AUC		log $1/IC_{50}$		CLOGP
	Obs	Calc[a]	Obs	Calc[b]	
1a	0.08	0.08	1.02	0.94	0.86
1b	1.12	1.18	1.16	1.48	1.76
1c	1.19	1.07	1.25	1.42	1.67
1d	1.39	1.75	1.51	1.76	2.23
1e	0.30	0.20	1.25	1.00	0.96
1f	–0.04	–0.07	0.80	0.86	0.73
2a	0.18	0.49	1.30	1.14	1.19
2b	1.80	1.58	2.00	1.68	2.09
2c	2.06	1.47	1.89	1.62	2.00
2d	1.99	2.16	2.01	1.96	2.56
2e	0.30	0.61	1.00	1.20	1.29
2f	0.48	0.33	0.92	1.06	1.06
3a	0.00	–0.06	1.30	1.10	1.48
3b	0.43	1.13	1.44	1.69	2.38
3c	1.26	1.01	1.28	1.63	2.29
3d	1.11	1.75	1.30	2.00[c]	2.85
3e	0.04	0.07	0.92	1.17	1.58
3f	0.08	–0.23	1.35	1.02	1.35
4a	0.18	0.38	1.39	1.32	1.81
4b	2.25	1.56	2.02	1.91	2.71
4c	2.24	1.44	2.02	1.85	2.62
4d	2.06	2.18	2.35	2.21	3.18
4e	0.30	0.51	1.47	1.38	1.91
4f	0.00	0.20	0.96	1.23	1.68
Lidocaine•HCl	2.00	–	1.80	–	

[a] Values calculated according to eq [1] (**1a–f** and **2a–f**) or eq [2] (**3a–f** and **4a–f**); [b] values calculated according to eq [3] (**1a–f** and **2a–f**) or eq [5] (**3a–c**, **3e**, **3f** and **4a–f**); [c] value predicted from eq [5] from whose derivation **3d** was omitted.

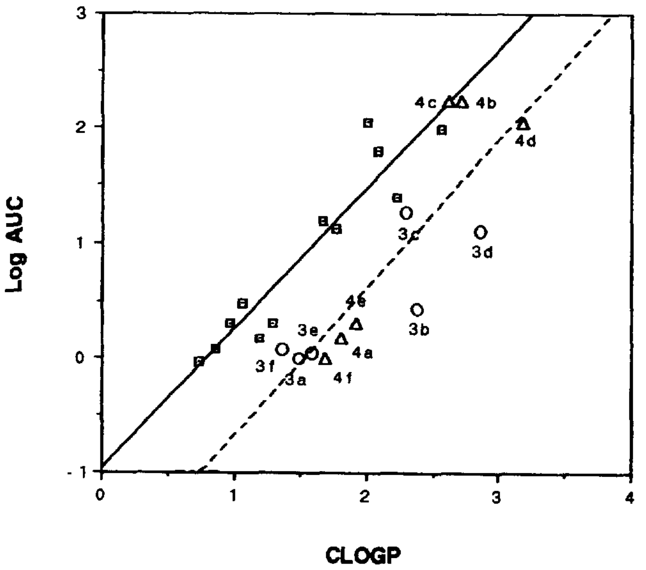


Fig 1. Plot of log AUC against CLOGP. Interpolation curves relative to eqs [1] and [2] are drawn as solid and dashed lines, respectively. Data points are labeled with squares (**1a–f** and **2a–f**), circles (**3a–f**) and triangles (**4a–f**).

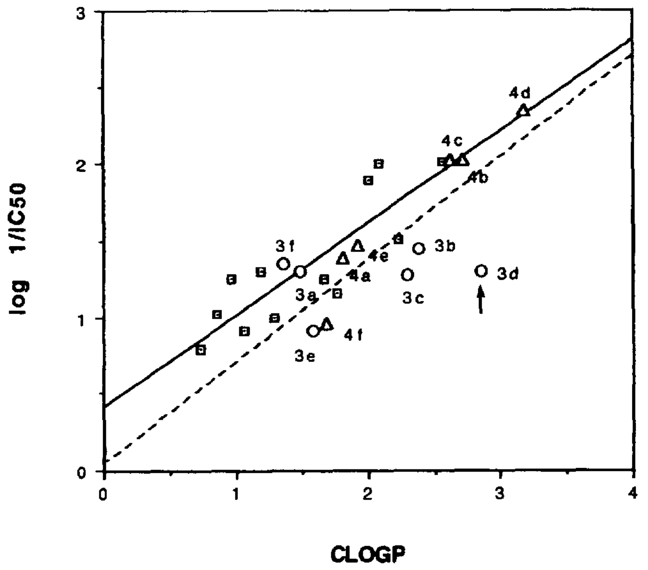


Fig 2. Plot of log $1/IC_{50}$ against CLOGP. Interpolation curves relative to eqs [3] and [5] are drawn as solid and dashed lines, respectively. Data points are labeled with squares (**1a–f** and **2a–f**), circles (**3a–f**) and triangles (**4a–f**). The arrow marks the outlier **3d** omitted from derivation of eq [5].

A comparison between the regression coefficients and intercepts of eqs [3] and [5] indicates that the two series of benzotriazoles, acetamides and isobutyramides, share a similar sensitivity of log $1/IC_{50}$ from CLOGP. In contrast to what was previously observed for the surface anesthesia, the branching of the side chain is not linked to a constant unfavorable effect on the infiltration anesthetic activity. However, it is also possible that in this assay pharmacokinetic phenomena take place to a larger extent, compared to the surface anesthesia test, which we could not parametrize.

Since compounds **4b**, **4c** and **4d** showed surface and infiltration anesthetic activities higher than those of the reference drug, we decided to test them in vivo on the rat sciatic nerve. The LD_{50}s (mg/kg) of the three compounds were also determined in mice and their therapeutic indices calculated as LD_{50}/IC_{50} ratio (IC_{50} refers to the mouse tail anesthesia and is expressed in mg/kg). The results of these assays are reported in table III together with those measured for lidocaine and for the previously investigated benzotriazole derivatives **2b**, **2c** and **2d**.

Compared with lidocaine, **4b** and **4c** exhibit a better performance in blocking the rat sciatic nerve (133 and 123 min for recovering of motor activity, respectively, versus 110 min for lidocaine). The duration of action of these two compounds is also longer than their corresponding acetamide homologs (**2b** and **2c**, with 103 and 90 min).

The isobutyramide derivative **4d** appears as the compound having the best overall pharmacological/toxicological profile (its therapeutic index is 9.4 whereas that of lidocaine is 5.1). Although the therapeutic index of **2b** is the highest (10.3), **4d** is significantly more active than **2b** in the surface and infiltration anesthesia assays (table IV). On the other hand, **4d** and **2b** are nearly equivalent in terms of duration of action in the sciatic nerve block test (table III).

Experimental protocols

Chemistry

Melting points were determined with a Kofler apparatus and are uncorrected. Microanalyses (C, H, N) are within ±0.4% of theoretical values. ^1H-NMR spectra were obtained with a Bruker WM-250 using CD_3OD as solvent.

Chemical shifts are expressed in ppm downfield from internal tetramethylsilane. Significant ^1H-NMR data are reported as follows: s singlet, t triplet, number of proton, coupling constants in hertz. Silica-gel UV-sensitive plates (Merck 60 F_{254}) and silica gel 40 (Merck 0.063–0.200 mm) were used for analytical TLC and column chromatography, respectively. Extracts were dried over sodium sulfate and solvents were removed under reduced pressure. All materials were obtained from commercial suppliers (Aldrich, Novabiochem) and used without further purification. Lidocaine•HCl was purchased from Sigma. The general method of synthesis described is illustrative of all analogous compounds.

General procedure for preparation of benzotriazolylisobutyric esters 5 and 6

To a magnetically stirred solution of sodium ethoxide (0.08 mol) in absolute ethanol, benzotriazole (0.08 mol) and ethyl-2-bromoisobutyrate (0.08 mol) were added successively. The reaction mixture was heated under reflux for 7 h and monitored by TLC. After cooling, the ethanol was removed under reduced pressure and the residue was treated with diethyl ether (100 ml), separated from the sodium bromide and washed with 2 N sodium hydroxide and water to remove the unreacted benzotriazole. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The obtained residue, containing 1- and 2-substituted isomers, was finally fractionated by column-chromatography using diethyl ether/*n*-hexane 7:3 v/v as eluent. Characterization of isolated products by UV and ^{1}H-NMR spectra showed that the first compound to be eluted was the 2-substituted benzotriazole (compound **5**: yield 40%, bp 145–148 °C, 0.7 mmHg; compound **6**: yield 47%, bp 120–125 °C, 0.3 mmHg).

General procedure for preparation of benzotriazolylisobutyric acids 7 and 8

The benzotriazolylisobutyric acids were obtained in quantitative yields by hydrolysis of the corresponding esters **5** and **6**. The ester derivative was suspended in 10% NaOH and the mixture was heated under reflux with magnetic stirring for 3 h. After cooling the reaction mixture was acidified with 1 N HCl to pH 1 and the product extracted with ethylacetate. The organic layer was washed with water, dried and evaporated. The crude compound **7** was crystallized from methanol and diethyl ether (mp 181–184 °C) whereas the crude compound **8** from water (mp 152–153 °C).

General procedure for preparation of benzotriazolylisobutyric amides 3a–f and 4a–f

To a solution of **7** or **8** (0.02 mol) in DMF (100 ml) cooled at 0 °C, were added successively the appropriate amino derivative (0.02 mol), 1-hydroxybenzotriazole (HOBt) (0.02 mol) and DCC (0.021 mol). The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. After this time *N,N'*-dicyclohexylurea (DCU) was filtered off; the resulting solution was diluted with dichloromethane (100 mL) and washed consecutively with brine, 2 N NaOH and brine. The organic layer was dried, filtered, and evaporated to dryness. The obtained residue was crystallized from the appropriate solvent or purified by column chromatography to give derivatives **3** or **4**, respectively, in yields ranging from 40 to 85%. The hydrochloride salts were prepared from the corresponding amines using dry HCl in anhydrous dichloromethane. Compounds **3f** and **4f** were obtained as dihydrochloride salts; the assigned formulae were supported by argentometric titration of the Cl ion.

Pharmacology

Corneal reflex test

Local anesthetic activity was evaluated in male New Zealand rabbits (Morini, S Polo d'Enza, Reggio Emilia, weighing 2.4–2.8 kg) as local surface anesthesia [6] evaluated by determining every 3 min the number of stimuli to the cornea, effected rhythmically with a Frey's horse-hair, necessary to produce the blink reflex. If the reflex did not occurr after 100 stimulations, anesthesia was considered total. At the beginning of the experiment care was taken to ascertain that this reflex was normal in both eyes of the rabbits. The aqueous solutions (2%) of the compounds studied were dropped onto the conjunctival sac so that the space between the eyelids contained a clearly visible film of solution for the set time of 3 min. Lidocaine solution (2%) was used for comparison.

Mouse tail anesthesia

This test was performed on male Swiss mice (Nossan, Correzzana, Milan, weighing 18–20 g) according to the method of Bianchi [7] in which the aqueous anesthetic solution (0.1 mL) is injected sc about 1 cm from the root of the tail. Fifteen minutes after injection, the pain reflex of all the injected animals was tested applying a small artery clip to the zone where the compound was injected. The proportion of animals that within 30 s did not show the usual pain reflex was noted for each dose. Lidocaine solutions were used for comparison. IC_{50} values were calculated for each compound by probit analysis using a computer program [8].

Rat sciatic nerve block

To better assess the local anesthetic activity and its duration of action, the rat sciatic nerve block test was performed [9]. Three groups of three male Wistar rats (Nossan, Correzzana, Milan, weighing 180–200 g) were used. Each rat received an injection (0.2 mL) of the aqueous anesthetic solution (2%) into the posterior aspect of the femur head. A positive effect of the drug resulted in a complete loss of motor control of the injected limb. In measuring the duration of the effect, the animals were observed from the time of onset of the motor paralysis at 5 min intervals for the first 30 min, and at 15 min intervals over the following time up to the first sign of motor activity.

Acute toxicity

The ip acute toxicity of the most active compounds **4b**, **4c** and **4d** was determined in male Swiss mice (Nossan, Correzzana, Milan, weighing 18–20 g) 7 days after treatment. LD_{50} values were calculated for each compound by probit analysis using a computer program [8].

QSAR studies

Calculation of overall octanol/water log P (CLOGP) values and regression analysis were performed by using the C-QSAR program [2]. The CLOGP values refer to non-ionized species. For correlation purposes, the biological data listed in table II were converted into logarithmic scales (table IV): log AUC (surface anesthetic activity) and log $1/IC_{50}$ (infiltration anesthetic activity). The 'leave-one-out' cross-validation method [5] was applied to evaluate the significance of the correlations. The *F*-values were calculated from the cross-validated residuals.

Acknowledgments

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