International Journal of Pharmaceutics, 75 (1991) 249–258

ADONIS 037851739100322K

IJP 02514

O,O'-(1,4-Xylylene)bispilocarpic acid esters as new potential double prodrugs of pilocarpine for improved ocular delivery. I. Synthesis and analysis

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(Received 18 March 1991)
(Accepted 9 May 1991)

Key words: Prodrug; Bispilocarpic acid diester; Synthesis; Analysis; Liquid chromatography; Thermospray liquid chromatography; Mass spectrometry; NMR

Summary

An analogue series representing novel diesters of bispilocarpic acid, *O,O'*-(1,4-xylylene) bispilocarpic acid esters, were synthesized as double prodrugs of pilocarpine in order to improve the ocular delivery characteristics of the drug. In previous studies, various bispilocarpic acid monoesters have been synthesized and evaluated as prodrugs of pilocarpine. However, these derivatives suffer from instability in aqueous solution and hence were not suitable as a pilocarpine prodrug. Based on earlier results, 1,4-xylylene bispilocarpate was selected as the starting material for the synthesis of diesters. Bispilocarpic acid diesters were prepared by esterifying the free hydroxyl groups of bispilocarpic acid monoesters. All the diesters formed a salt with 3 equivalents of fumaric acid. The yields of bispilocarpic acid diester fumarates varied from 48 to 99%. The identification of the compounds and evaluation of the purity of synthetic products were performed by liquid chromatography with UV detection, thermospray liquid chromatography mass spectrometry, electron impact ionization mass spectrometry and ^{1}H-NMR spectroscopy. The elemental composition of each compound was determined on high-resolution mass spectrometry by measuring the accurate mass of the molecular ion.

Introduction

The concept of a prodrug was first used in 1958 to describe compounds which undergo biotransformation within the body prior to exerting their pharmacological action (Balant et al., 1990). Since then, prodrugs have been extensively studied in order to find compounds that mask bad taste/odour, improve aqueous solubility and chemical stability, reduce toxicity, and increase gastrointestinal, transdermal and ocular drug absorption (Sinkula and Yalkowsky, 1975; Stella et al., 1985; Bundgaard, 1987).

The notion of a double prodrug has been used in describing the structure of a prodrug, which has been prepared from a prodrug (Bundgaard,

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1989). Double prodrugs are used in order to overcome the drawbacks of the usual prodrugs, e.g. lability in aqueous solution (Bundgaard et al., 1986a).

Pilocarpine is widely used clinically for the control of glaucoma. Due to its rapid precorneal elimination and low lipophilicity its ocular bioavailability is low: only 1–3% or less of instilled pilocarpine gains access to intraocular tissues (Chrai and Robinson, 1974; Lazare and Horlington, 1975). Consequently, concentrated pilocarpine eye-drops must be administered several times daily and frequently this causes ocular and more rarely systemic side-effects (Stafford, 1981). These factors impair patient compliance (Norell, 1980).

The prodrug approach has been applied in order to improve the delivery characteristics of pilocarpine. Previously described prodrug types include quaternary ammonium salts of pilocarpine (Bodor, 1977), pilocarpic acid monoesters (Bundgaard et al., 1986a), pilocarpic acid diesters (Bundgaard et al., 1986b), bispilocarpic acid monoesters (Järvinen et al., 1991b,f) and *O,O'*-dicarboxylate (dibenzyl) bispilocarpates (Järvinen et al., 1991c,d). Even though the first reports on the quaternary derivatives of pilocarpine appeared in 1977, no studies have subsequently been published nor has any investigation been carried out. Pilocarpic acid and bispilocarpic acid monoesters are insufficiently stable in aqueous solutions to permit formulations of eye-drops with an adequate shelf-life to be produced. In contrast, double prodrugs of pilocarpine, pilocarpic acid diesters showed good stability in aqueous solutions (Bundgaard et al., 1986a; Järvinen et al., 1991e). These prodrugs also increased the ocular bioavailability of pilocarpine in rabbits (Mosher et al., 1987). In spite of the promising nature of pilocarpic acid diesters, their low aqueous solubility and ocular irritation may hinder the clinical use of these derivatives (Mosher, 1986). The previously described bispilocarpic acid diesters, *O,O'*-dicarboxylate (dibenzyl) bispilocarpates, may yield some potential prodrug derivatives on the basis of recent in vitro results (Järvinen et al., 1991d).

The purpose of the present study was to prepare water-soluble, double prodrug derivatives of pilocarpine, *O,O'*-(1,4-xylylene) bispilocarpic acid esters, from the bispilocarpic acid monoesters reported earlier, 1,4-xylylene bispilocarpate (Järvinen et al., 1991b,f). The proportion of the promoiety of bispilocarpic acid is minimized in order to reduce irritation, the main drawback of pilocarpic acid diesters (Mosher, 1986). This paper also describes methods for the identification and evaluation of the purity of the synthetic products by liquid chromatography with UV detection (LC-UV), thermospray liquid chromatography mass spectrometry (TSP-LC-MS), electron impact ionization mass spectrometry (EI-MS) and ^{1}H-NMR spectroscopy. The elemental compositions of the synthesized compounds were determined by accurately measuring the mass via high-resolution mass spectrometry (HR-MS).

Materials and Methods

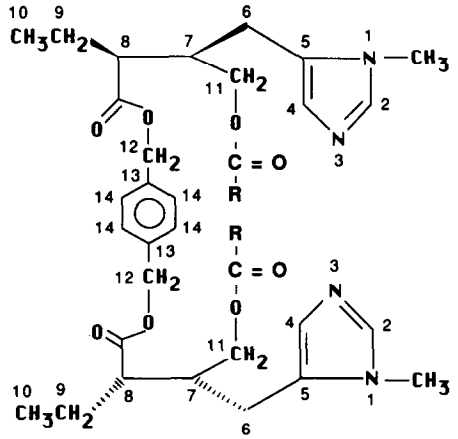
Chemicals

Pilocarpine hydrochloride was kindly provided by Huhtamäki Oy Leiras (Tampere, Finland). Acetyl chloride, propionyl chloride, butyryl chloride, valeryl chloride, acetoxyacetyl chloride, cyclopropanecarbonyl chloride, benzoyl chloride, fumaric acid, and calcium sulphate were obtained from Aldrich (Steinheim, Germany). Toluene, petroleum ether, 2-propanol, monobasic potassium phosphate, potassium carbonate, and ammonium acetate were purchased from Merck (Darmstadt, Germany). Methanol (HPLC grade) and acetonitrile (HPLC grade) were from Baker (Deventer, The Netherlands).

Synthesis of bispilocarpic acid diester

The *O,O'*-(1,4-xylylene) bispilocarpic acid esters, i.e. bispilocarpic acid diesters, **1–7** (Fig. 1), were prepared by esterifying bispilocarpic acid monoester, 1,4-xylylene bispilocarpate, with appropriate acid chloride. 1,4-xylylene bispilocarpate was synthesized as described in Järvinen et al. (1991b).

O,O'-Diacetyl (1,4-xylylene)bispilocarpate fumarate (compound 1) Acetyl chloride (767 mg; 9.77 mmol) was added dropwise during a period



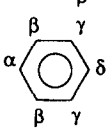
	R
1.	$-\overset{\alpha}{C}H_3$
2.	$-\overset{\alpha}{C}H_2\overset{\beta}{C}H_3$
3.	$-\overset{\alpha}{C}H_2\overset{\beta}{C}H_2\overset{\gamma}{C}H_3$
4.	$-\overset{\alpha}{C}H_2\overset{\beta}{C}H_2\overset{\gamma}{C}H_2\overset{\delta}{C}H_3$
5.	$-\overset{\alpha}{C}H_2\overset{\beta}{C}OOCH_3$
6.	
7.	

Fig. 1. Structures of the *O,O'*-(1,4-xylylene) bispilocarpic acid esters, i.e. bispilocarpic acid diesters, studied.

of 30–40 h to a mixture of 1,4-xylylene bispilocarpate (679 mg; 1.22 mmol) and potassium carbonate (2026 mg; 14.66 mmol) in toluene (150 ml). An aqueous solution of sodium bicarbonate (2%, 150 ml) was added to the reaction mixture which was then stirred at room temperature for 3 h. The layers were separated and the toluene phase was washed twice with distilled water (2 × 150 ml), dried on calcium sulphate (30 min) and evaporated under reduced pressure to give oily *O,O'*-diacetyl (1,4-xylylene) bispilocarpate as free base, 780 mg (1.22 mmol). The oil was dissolved in toluene (20 ml) and a solution of fumaric acid (425 mg; 3.66 mmol) in 2-propanol (10 ml) was added. The salt was precipitated with petroleum ether. After standing overnight *O,O'*-diacetyl (1,4-xylylene) bispilocarpate fumarate crystals were isolated (750 mg; 0.76 mmol). The yield was 62%, m.p. 58–60 °C (fumarate), $n_d^{20} = 1.5230$ (free base). Formula $C_{46}H_{58}O_{20}N_4$ (fumarate salt).

O,O'-Dipropionyl (1,4-xylylene) bispilocarpate fumarate (compound 2) The diester was prepared from 1,4-xylylene bispilocarpate (473 mg; 0.85 mmol) and propionyl chloride (631 mg; 6.82 mmol) according to the procedure described above to give 626 mg (0.94 mmol) of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. The *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate was crystallized from toluene/petroleum ether to give 730 mg (0.72 mmol) of diester fumarate. The yield was 85%, m.p. 86–89 °C, $n_d^{20} = 1.5205$. Formula $C_{48}H_{62}O_{20}N_4$ (fumarate salt).

O,O'-Dibutyryl (1,4-xylylene) bispilocarpate fumarate (compound 3) The diester was prepared from 1,4-xylylene bispilocarpate (431 mg; 0.78 mmol) and butyryl chloride (633 mg; 6.22 mmol) via the procedure described earlier to give 617 mg (0.89 mmol) of *O,O'*-dibutyryl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. The *O,O'*-dibutyryl (1,4-xylylene) bispilocarpate fumarate was crystallized from toluene/petroleum ether to give 800 mg (0.77 mmol) of diester fumarate. The yield was 99%, m.p. 90–92 °C, $n_d^{20} = 1.5070$. Formula $C_{50}H_{66}O_{20}N_4$ (fumarate salt).

O,O'-Divaleryl (1,4-xylylene) bispilocarpate fumarate (compound 4) The diester was prepared from 1,4-xylylene bispilocarpate (395 mg; 0.71 mmol) and valeryl chloride (685 mg; 5.68 mmol) by the above procedure to give 479 mg (0.66 mmol) of *O,O'*-divaleryl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. *O,O'*-divaleryl (1,4-xylylene) bispilocarpate fumarate was crystallized from toluene/petroleum ether to give 567 mg (0.53 mmol) of diester fumarate. The

yield was 75%, m.p. 84–86 °C, $n_d^{20} = 1.5080$. Formula $C_{52}H_{70}O_{20}N_4$ (fumarate salt).

O,O'-Diacetoxyacetyl (1,4-xylylene) bispilocarpate fumarate (compound 5) The diester was prepared from 1,4-xylylene bispilocarpate (450 mg; 0.81 mmol) and acetoxyacetyl chloride (886 mg; 6.49 mmol) by the earlier described procedure to give 713 mg (0.95 mmol) of *O,O'*-diacetoxyacetyl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. *O,O'*-Diacetoxyacetyl (1,4-xylylene) bispilocarpate fumarate was crystallized from toluene/petroleum ether to give 873 mg (0.79 mmol) of diester fumarate. The yield was 98%, m.p. 84–86 °C, $n_d^{20} = 1.5182$. Formula $C_{50}H_{62}O_{24}N_4$ (fumarate salt).

O,O'-Dicyclopropanecarbonyl (1,4-xylylene) bispilocarpate fumarate (compound 6) The diester was prepared from 1,4-xylylene bispilocarpate (547 mg; 0.99 mmol) and cyclopropanecarbonyl chloride (788 mg; 7.92 mmol) by the previously described procedure to give 686 mg (0.99 mmol) of *O,O'*-dicyclopropanecarbonyl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. The diester was crystallized from toluene/petroleum ether to give 843 mg (0.81 mmol) of diester fumarate. The yield was 82%, m.p. 73–75 °C, $n_d^{20} = 1.5290$. Formula $C_{50}H_{62}O_{20}N_4$ (fumarate salt).

O,O'-Dibenzoyl (1,4-xylylene) bispilocarpate fumarate (compound 7) The diester was prepared from 1,4-xylylene bispilocarpate (486 mg; 0.88 mmol) and benzoyl chloride (990 mg; 7.04 mmol) by the described procedure to give 695 mg (0.91 mmol) of *O,O'*-dibenzoyl (1,4-xylylene) bispilocarpate as a free base. The compound formed salt with 3 equivalents of fumaric acid. *O,O'*-Dibenzoyl (1,4-xylylene) bispilocarpate fumarate was crystallized from toluene/petroleum ether to give 464 mg (0.42 mmol) of diester fumarate. The yield was 48%, m.p. 72–75 °C, $n_d^{20} = 1.5605$. Formula $C_{56}H_{62}O_{20}N_4$ (fumarate salt).

Melting points

The uncorrected melting points for bispilocarpic acid diesters were determined using a Reichert thermovar (Wien, Austria) apparatus.

Index of refraction

The index of refraction for bispilocarpic acid diesters as a free base was measured at room temperature with an Atago Illuminator (Japan).

Liquid chromatography

Liquid chromatography (LC) was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman programmable UV detector 166 (set at 215 nm), System gold data module (Beckman Instruments Inc., San Ramon, U.S.A.), Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with column thermostat and Rheodyne 7080-080 loop (20 μl) injector. A deactivated Supelcosil LC8-DB (15 cm × 4.6 mm i.d., 5 μm) reversed-phase column (Supelco, Bellefonte, U.S.A.) was used as a stationary phase. The mobile phase was a mixture of methanol and 0.02 M KH_2PO_4 (pH 4.5), which was individually optimized for each compound. The flow rate was 1.0 ml/min.

Thermospray liquid chromatography-mass spectrometry

Thermospray liquid chromatography-mass spectrometry (TSP-LC-MS) applications described here were carried out on a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, U.K.) equipped with a thermospray/plasmaspray interface probe. The instrument was operated in the thermospray ionization mode. The thermospray probe temperature was adjusted to 190 °C, the ion source temperature was 150 °C, the repeller voltage was 220 V and the other ion source conditions were optimized daily. The LC system connected to the inlet of the thermospray interface consisted of a Beckman model 112 pump (Beckman Instruments Inc., San Ramon, U.S.A.) for solvent delivery and the samples were injected with a Rheodyne 7125 injector (loop volume 20 μl). The compounds were separated using a deactivated Supelcosil LC8-DB reversed-phase column (15 cm × 4.6 mm i.d., 5 μm) with an isocratic solvent system of 0.2 M ammonium acetate-acetonitrile (32–68%) at a flow rate of 1.0 ml/min.

In flow injection, a sample was introduced without chromatographic separation to the thermospray source.

Electron impact ionization mass spectrometry

The positive electron impact ionization (EI) mass spectra of the bispilocarpic acid diesters were recorded on a VG 70-250SE magnetic sector mass spectrometer (VG Analytical, Manchester, U.K.). All EI spectra were recorded under the following conditions: electron energy, 70 eV; ionization current, 500 μA; ion source temperature, 150 °C. The resolution of the instrument was adjusted to 10000. Samples were introduced into the mass spectrometer in a glass sample holder using a direct insertion probe. The probe temperature was raised from 30 to 500 °C at a rate of 100 °C/min.

Accurate measurement of mass for molecular ions was carried out under the described conditions using perfluorokerosene as a reference compound.

NMR spectroscopy

^{1}H spectra were recorded on a Bruker AM-250 FT/ASPECT 3000 spectrometer using a 5 mm ^{1}H/^{13}C dual probe, operating at 250.134 MHz for ^{1}H measurements. To 0.6 ml of CD_3OD with Me_4Si (0.1%) as an internal standard was added 20–40 mg of the desired bispilocarpic acid diester. The number of data points in the ^{1}H experiment was 32 K, total relaxation time 16 s, number of scans 128 and pulse angle 30 °.

Results and Discussion

Synthesis of bispilocarpic acid diesters

The *O,O'*-(1,4-xylylene) bispilocarpic acid esters, i.e. bispilocarpic acid diesters, are novel double prodrugs of pilocarpine. The spacer chain between pilocarpic acid was selected on the basis of previous findings (Järvinen et al., 1991b,f) where the synthesis and properties of various bispilocarpic acid monoesters have been described.

The yields from synthesis of these bispilocarpic acid diester fumarates were relatively high, varying from 48 to 99%. All bispilocarpic acid diesters formed salts with 3 equivalents of fumaric acid. Since pilocarpic acid diesters are known to form salts with 1.5 equivalents of fumaric acid (Bundgaard et al., 1985, 1986b; Järvinen et al., 1991a), the formation of a salt may follow the same principle in both structures.

Typical impurities present in the synthetic product were toluene, incompletely esterified bispilocarpic acid diesters and pilocarpic acid diesters. Due to its high boiling point, toluene remains in the crystals despite careful drying. The low melting points and hygroscopic nature of bispilocarpic acid diesters make drying of the crystals very difficult. Incompletely esterified bispilocarpic acid diesters were observed when excess acid chloride was not used in esterification. A major impurity in bispilocarpic acid monoesters, pilocarpic acid monoester (Järvinen et al., 1991b), leads to the formation of pilocarpic acid diester, which was a major impurity in the synthesis of the bispilocarpic acid diesters.

The estimated purities of the synthesized compounds ranged from 75 to 95%, being most often about 90% (w/w). The estimated purities (in %) of the compounds are based on NMR determinations.

Analysis by liquid chromatography

A reversed-phase LC procedure was developed to provide a suitable method for evaluating the purity of the synthetic products. The LC-UV procedure is also a simple and rapid method for the determination of physicochemical properties.

Peak tailing is often encountered as a problem in the analysis of the basic compounds (Köhler et al., 1986; Bayer and Paulus, 1987). A tailing factor greater than 1.5 can be a reason for rejecting a chromatographic method. The tailing problem has been partly abolished by the use of paired ion techniques (Bidlingmeyer, 1980) and end-capping columns (Cooke and Olsen, 1980). The use of a Supelco RP-8-DB column resulted in symmetric peaks without addition of triethylamine to the eluent. A typical LC-UV chromatogram of the synthetic product (*O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate; compound **2**) is shown in Fig. 2. The tailing factor for this com-

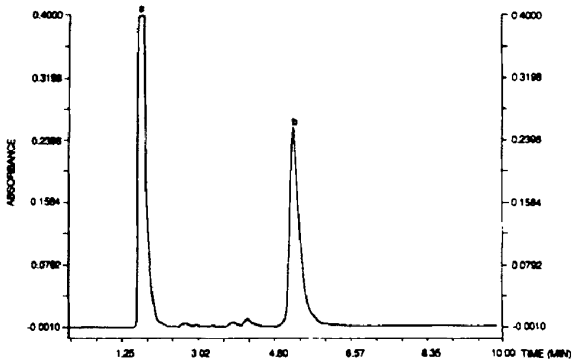


Fig. 2. LC-UV chromatogram of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate. Peaks: (a) fumaric acid; (b) diester as a free base. LC conditions are described in the text.

pound is 1.3, which is less than the minimum requirement of 1.5.

Thermospray liquid chromatography mass spectrometry (TSP-LC-MS) was employed for the identification of the peaks after liquid chromatographic separation and to obtain TSP-mass spectra of the compounds by flow-injection.

The TSP-mass spectrum (flow injection) of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate is shown in Fig. 3a. The spectrum displays a base peak at m/z 667, which represents the protonated molecular ion $[M + H]^+$ of the free diester base. Two other abundant quasi-molecular ions, m/z 705 $[M + 39]^+$ and m/z 689 [M +

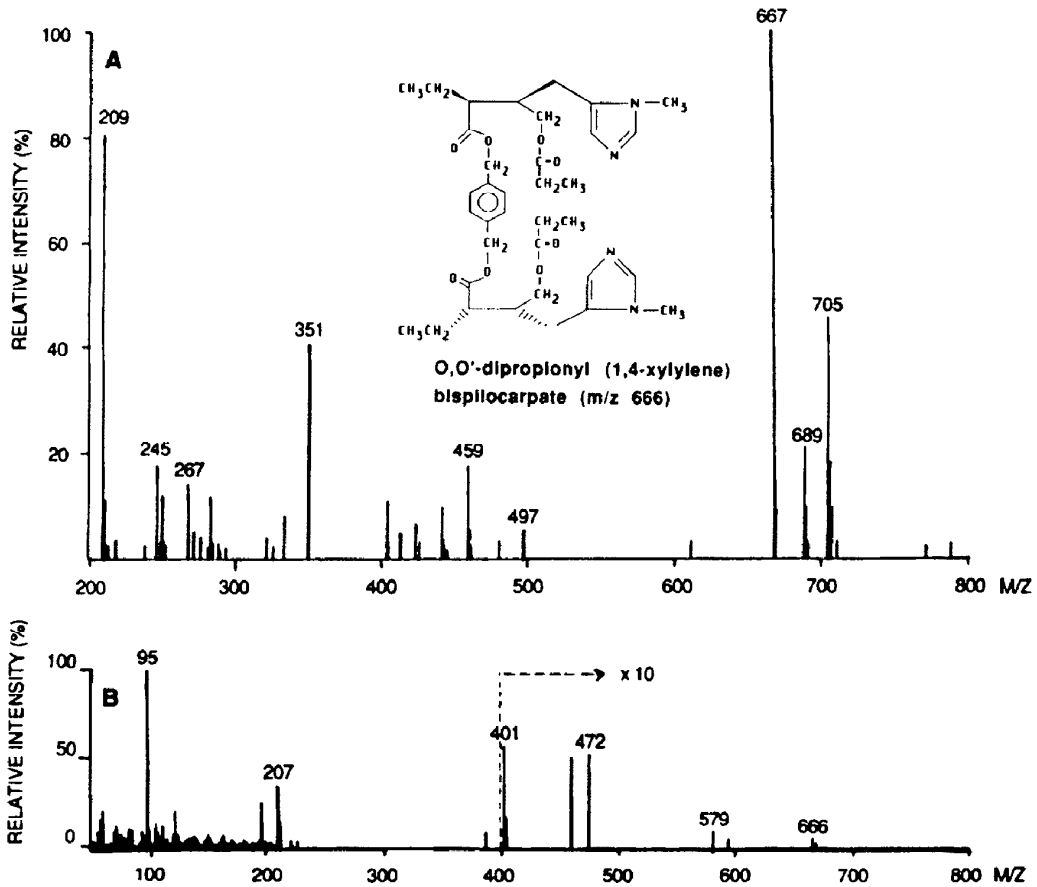


Fig. 3. (A) Thermospray mass spectrum of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate. (B) Electron impact ionization (70 eV) mass spectrum of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate.

TABLE 1

Relative abundances of the ions in the TSP mass spectra of bispilocarpic acid diesters

Compound	$[M + H]^+$ (%)	$[M + 39]^+$	$[M + 23]^+$	Other characteristic fragment ions (%)
1	639 (100%)	677 (35%)	661 (21%)	469 (12%), 431 (50%), 389 (37%), 209 (55%)
2	667 (100%)	705 (46%)	689 (22%)	459 (18%), 404 (11%), 351 (40%), 209 (80%)
3	695 (100%)	732 (77%)	717 (36%)	487 (13%), 455 (8%), 417 (14%), 209 (28%)
4	723 (100%)	761 (86%)	745 (33%)	431 (9%), 209 (35%), – –
5	755 (23%)	792 (47%)	777 (55%)	547 (12%), 484 (18%), 447 (27%), 209 (100%)
6	691 (100%)	728 (89%)	713 (42%)	480 (30%), 453 (32%), 415 (46%), 209 (73%)
7	763 (100%)	–	785 (28%)	555 (21%), 489 (7%), 451 (8%), 209 (42%)

23]$^+$, may be due to the cationized molecular ions, $[M + K]^+$ and $[M + Na]^+$, respectively (Maeder, 1990). The major fragment ions can be seen at m/z 459, m/z 404, m/z 351 and m/z 209. The fragment of m/z 404 is postulated to be the derivative resulting from the cleavage of the

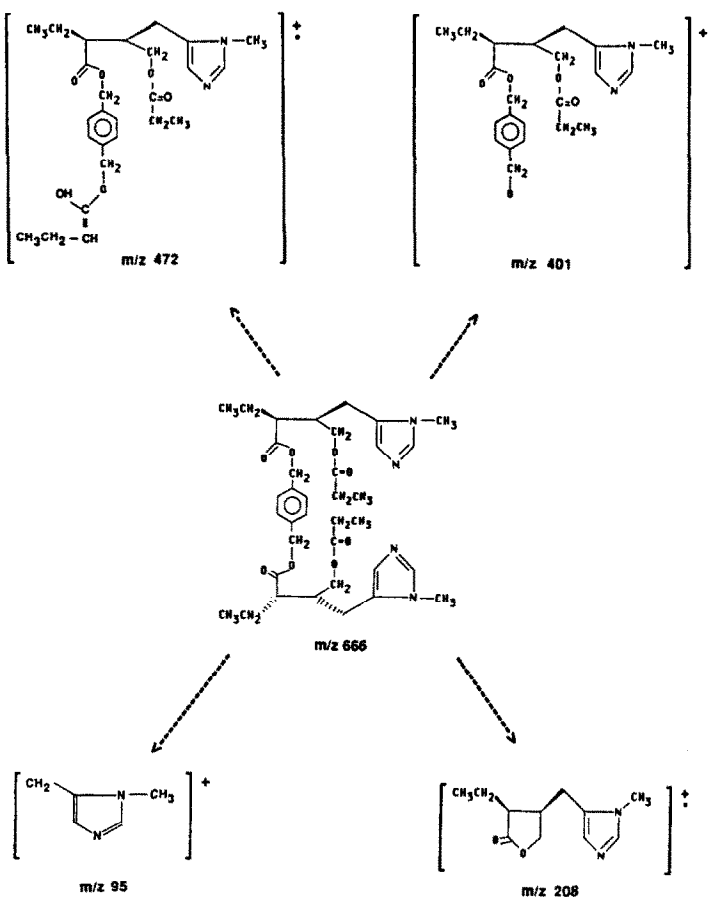


Fig. 4. Structures of the most important fragment ions of *O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate in the electron impact ionization mass spectrum.

TABLE 2

Relative abundances of the ions in the EI mass spectra of bispilocarpic acid diesters

Compound	$[M^{+\cdot}]$	Other fragment ions
1	638 (5%)	458 (22%), 443 (19%), 387 (24%), 208 (21%), 207 (45%), 96 (20%), 95 (100%)
2	666 (5%)	472 (53%), 457 (45%), 401 (58%), 208 (13%), 207 (34%), 96 (43%), 95 (100%)
3	694 (3%)	486 (26%), 471 (14%), 415 (17%), 208 (20%), 207 (37%), 96 (26%), 95 (100%)
4	722 (9%)	500 (60%), 485 (31%), 429 (41%), 208 (17%), 207 (34%), 96 (46%), 95 (100%)
5	–	325 (21%), 239 (13%), 208 (19%), 207 (73%), 96 (27%), 95 (100%), –
6	690 (5%)	484 (36%), 469 (19%), 413 (30%), 208 (16%), 207 (46%), 96 (30%), 95 (100%)
7	762 (1%)	243 (10%), 208 (15%), 207 (38%), 96 (26%), 95 (100%) – –

first pilocarpine molecule. The ion at m/z 209 [pilocarpine + H] may be formed by fragmentation of the structure following the cyclization of the ion to pilocarpine or by hydrolysis of the molecule in the heated capillary with cyclization to pilocarpine. The TSP-mass spectra (flow-injection) of all the other bispilocarpic acid diesters studied show corresponding fragment ions. The relative abundances of the selected ions are summarized in Table 1.

The TSP-mass spectrum from the diester peak after reversed-phase chromatographic separation is almost identical to that in flow-injection. However, a perceptible peak broadening was achieved for bispilocarpic acid diesters and hence better mass spectra were observed by flow injection.

Electron impact ionization mass spectrometry

Electron impact ionization mass spectrometry (EI-MS) was performed to obtain structural information on the bispilocarpic acid diesters and to make accurate measurements of the mass of the molecular ions in order to determine the elemental composition of the compounds.

A typical mass spectrum (70 eV) of a bispilocarpic acid diester (*O,O'*-dipropionyl (1,4-xylylene) bispilocarpate fumarate) is shown in Fig. 3b and the structures of the fragment ions are illustrated in Fig. 4. The spectrum shows a molecular ion $[M]^{+\cdot}$ of the free base at m/z 666 with very low intensity. The ion at m/z 472 may be formed by McLafferty rearrangement (Davis, 1987) and that of m/z 401 by cleavage of the other ester moiety in the spacer chain. The fragment ion at m/z 207 and 208 may correspond to the pilocarpine moiety. These ions may also originate from the decomposition of the prodrug on the heated sample probe. Fragmentation of pilocarpine may involve cleavage of the imidazole ring to give a base peak at m/z 95. Low intensities of molecular ions were typical for all bispilocarpic acid diesters and for *O,O'*-diacetoxyacetyl

TABLE 3

Measured and calculated accurate mass of bispilocarpic acid diesters

Compound	Observed mass	Calculated mass	Error (mmu)	Elemental composition (free base)
1	638.32739	638.33156	4.2	$C_{34}H_{46}O_8N_4$
2	666.35667	666.36296	6.2	$C_{36}H_{50}O_8N_4$
3	694.39378	694.39415	0.4	$C_{38}H_{54}O_8N_4$
4	722.42308	722.42546	2.4	$C_{40}H_{58}O_8N_4$
5	– [a]	–	–	
6	690.36014	690.36286	2.7	$C_{38}H_{50}O_8N_4$
7	762.36774	762.36286	4.9	$C_{44}H_{50}O_8N_4$

[a] $[M^{+\cdot}]$ peak was not observed.

TABLE 4

Proton chemical shifts for compounds 1–7 (ppm)

Proton	Compound						
	1	**2**	**3**	**4**	**5**	**6**	**7**
N-Me	3.71	3.69	3.72	3.69	3.71	3.73	3.71
2	8.46	8.39	8.51	8.35	8.52	8.55	8.41
4	7.19	7.15	7.21	7.13	7.22	7.24	7.20
6	2.71	2.71	2.72	2.70	2.70	2.74	2.82
7	2.36	2.36	2.36	2.36	2.36	2.38	2.53
8	2.56	2.55	2.55	2.55	2.54	2.57	2.65
9	1.69	1.69	1.69	1.69	1.69	1.70	1.75
10	0.86	0.86	0.86	0.86	0.86	0.87	0.89
11	4.06	4.07	4.07	4.07	4.07	4.08	4.32
12	5.15	5.15	5.15	5.15	5.16	5.16	5.07
14	7.41	7.41	7.41	7.41	7.42	7.41	7.32
R_α	2.00	2.30	2.27	2.29	2.11	1.69	–
R_β	–	1.08	1.59	1.55	4.58	0.86	7.93
R_γ	–	–	0.91	1.33	–	–	7.46
R_δ	–	–	–	0.91	–	–	7.60
Fumarate	6.71	6.72	6.71	6.72	6.72	6.71	6.71

(1,4-xylylene) bispilocarpate (compound **5**) no molecular ion was observed. The most prominent fragment ions with respect to relative intensity of all the bispilocarpic acid diester fumarates studied are listed in Table 2.

The elemental compositions of the molecular ions were determined by accurately measuring the mass via high-resolution mass spectrometry. The molecular peak for *O,O'*-diacetoxyacetyl (1,4-xylylene) bispilocarpate did not appear even when low electron energy (20 eV) was used. The error between the observed and calculated masses was below 6.2 mmu for all the bispilocarpic acid diesters studied (Table 3). This reliably verifies the elemental composition of the prepared bispilocarpic acid diesters.

NMR spectroscopy

NMR spectroscopy has occupied an important position amongst the various physical techniques employed for the identification and documentation of drug substances. In Table 4 we report all the important ^1H chemical shifts for compounds **1–7**. The ^1H shifts from structures **1–7** were assigned based on the data from a previous article by Järvinen et al. (1990a) and clearly indicate good agreement with the chemical shifts and structures.

The limit of detection of impurities and solvent residues was lower than 0.5 mol%. Comparing integrals from $COOCH_2$-Ar and α protons in side chain R, the degree of bisesterification was determined. Other impurities were identified and quantitated using the signals from the methyl group in the ethyl (CH_3CH_2-) side chain. No significant degradation products were observed during NMR measurements.

Conclusions

A series of various *O,O'*-(1,4-xylylene) bispilocarpic acid esters (i.e. bispilocarpic acid diesters), a new class of pilocarpine double prodrug, were synthesized with the aim of preparing potentially useful prodrug derivatives in reasonable yield. The free hydroxyl group of the bispilocarpic acid monoester was blocked with the appropriate acid chloride to obtain the bispilocarpic acid diesters. All the derivatives formed a salt with 3 equivalents of fumaric acid. The above-described methods, LC-UV, LC-TSP-MS, EI-MS and NMR, allow the rapid and unambiguous identification and purity evaluation of the derivatives. HR-MS was employed for the accurate determination of the mass of the molecular ions and, based on these

results, the elemental compositions of the compounds were evaluated.

Acknowledgements

The authors would like to thank Huhtamäki OY Leiras (Finland), the Technology Development Centre (Finland), Jenny and Antti Wihuri Foundation and the Finnish Cultural Foundation for financial support. We also thank Leiras for research collaboration. We are grateful to Mr Jukka Knuutinen and Ms Päivi Varis for skillful technical assistance.

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