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Synthesis and analysis of *O,O'*-dicarboxylate (dibenzyl) bispilocarpates as possible prodrugs of pilocarpine

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Abstract: As a part of a series of studies to develop prodrug derivatives of pilocarpine, the *O,O'*-succinyl (dibenzyl), *O,O'*-glutaryl (dibenzyl), *O,O'*-adipoyl (dibenzyl), *O,O'*-fumaryl (dibenzyl), and *O,O'*-terephthaloyl (dibenzyl) bispilocarpate fumarates were synthesized as a new class of pilocarpine prodrugs. The compounds were prepared from pilocarpic acid benzyl monoester by coupling two pilocarpic acid benzyl monoesters together with spacer chains by usual esterification methods. Liquid chromatography, thermospray liquid chromatography–mass spectrometry, high-resolution mass spectrometry, and NMR spectroscopy were applied to the identification and the purity evaluation of the synthetic products.

Keywords: *Pilocarpine; prodrug; bispilocarpic acid diester; synthesis; analysis.*

Introduction

In many cases, undesirable delivery properties of a drug may be difficult to overcome with conventional pharmaceutical formulations. Undesirable drug properties can be changed by reversible derivatives of parent drug, i.e. prodrugs [1–3]. Prodrugs have been designed to abolish drug undesirable properties such as poor aqueous solubility [4, 5], lability in aqueous solutions [6], poor dermal delivery [7–9], poor oral absorption [10], low ocular bioavailability [11], and systemic absorption of ocularly topically applied drugs [12].

Pilocarpine is widely used for controlling the elevated intraocular pressure associated with glaucoma. However, its ocular bioavailability is very low. This can be ascribed to rapid loss of the drug from precorneal area [13], conjunctival absorption [14], and poor corneal penetration [15]. Because of poor ocular bioavailability and rapid elimination from the eye, concentrated pilocarpine eye-drops must be administered into the eye several times a day causing ocular and systemic side-effects [16] and reduced patient compliance [17].

These drawbacks of pilocarpine may probably be overcome by using the prodrug approach, because delivery problems are in part dependent upon physicochemical properties, e.g. lipophilicity of the drug [18]. Bundgaard *et al.* have described pilocarpic acid monoesters [19] and pilocarpic acid diesters [20] as prodrugs of pilocarpine. Pilocarpic acid diesters have been reported to be more lipophilic than the parent drug, stable in aqueous solutions and, susceptible to enzymatic conversion to pilocarpine in the presence of esterase enzymes [20, 21].

The purpose of the present study was to prepare a new class of pilocarpine prodrugs, *O,O'*-dicarboxylate (dibenzyl) bispilocarpates, i.e. bispilocarpic acid diesters. The amount of pro-moiety in these new pilocarpine prodrugs is minimized without losing the desirable properties of earlier described pilocarpic acid diesters. This paper describes also the methods for 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), high-resolution mass spectrometry (HR-MS) and ^{1}H and ^{13}C NMR spectroscopy.

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Materials and Methods

Reagents and chemicals

Pilocarpine hydrochloride was supplied by Huhtamäki Oy Leiras (Finland). Succinyl chloride, glutaryl chloride, adipoly chloride, fumaryl chloride, terephthaloyl chloride, fumaric acid, and calcium sulphate were obtained from Aldrich (Steinheim, Germany). Chloroform, 2-propanol, petroleum ether, ethyl acetate, toluene, monobasic potassium phosphate, and potassium carbonate were purchased from Merck (Darmstadt, Germany). Dimethylsulphoxide and benzyl chloride were from Merck (Munich, Germany). Methanol (HPLC-grade) and acetonitrile (HPLC-grade) were from Baker (Deventer, The Netherlands).

Synthesis of pilocarpic acid

The sodium salt of pilocarpic acid (sodium pilocarpate) was synthesized as described previously [22].

Synthesis of pilocarpic acid benzyl monoester

The synthesis of pilocarpic acid benzyl monoester was modified from the earlier described procedure [19]. The synthesis was started by dissolving 8.00 mmol of sodium pilocarpate in 60 ml dimethylsulphoxide. To the solution 8.00 mmol of benzyl chloride was added drop-wise during 1 h. The solution was stirred at room temperature for 24–72 h and then poured into 100 ml of distilled water. The mixture was extracted with two 100 ml portions of ethyl acetate and the combined ethyl acetate extracts were washed with 150 ml of distilled water, 150 ml of 2% sodium bicarbonate and 150 ml of distilled water, sequentially. The ethyl acetate extracts were dried over calcium sulphate and the solvent was removed under reduced pressure to give pilocarpic acid benzyl monoester. The monoester was recrystallized from chloroform/petroleum ether to give 1125 mg (3.56 mmol) of pilocarpic acid benzyl monoester, m.p. 101–104°C (lit. [19] m.p. 84–85°C).

Synthesis of bispilocarpic acid diesters

O,O′-*succinyl (dibenzyl) bispilocarpate fumarate (compound 1)*

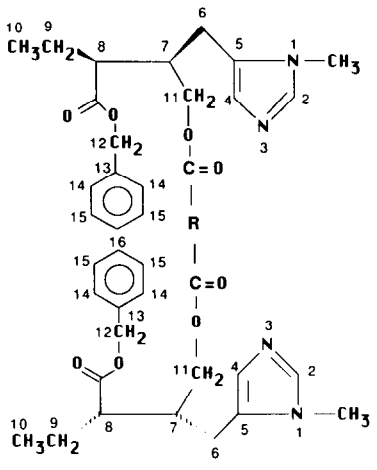
To a mixture of 633 mg (2.00 mmol) pilocarpic acid benzyl monoester and 553 mg (4.00 mmol) of potassium carbonate in toluene (40 ml) was added drop-wise 124 mg (0.80 mmol) of succinyl chloride during 24 h. The solution was stirred at room temperature for about 48 h. To the reaction mixture was added 2% aqueous solution of sodium bicarbonate (40 ml) and the mixture was stirred at room temperature for 3 h. The layers were separated and the toluene phase was washed twice with distilled water (2 × 50 ml), dried with calcium sulphate (30 min) and evaporated under reduced pressure to give *O,O′*-succinyl (dibenzyl) bispilocarpine (free base) as oil 437 mg (0.61 mmol). The oil was dissolved in toluene (15 ml) and a solution of fumaric acid (212 mg, 1.83 mmol) in 2-propanol (5 ml) was added. The compound formed a salt with three equivalents of fumaric acid which was precipitated with petroleum ether. After storing overnight the fumarate salt of *O,O′*-succinyl (dibenzyl) bispilocarpate was isolated to give 402 mg (0.38 mmol) of bispilocarpic acid diester fumarate. The yield was 47%, m.p. 65–67°C, $\phi_d^{20} = 1.5360$ (free base). Formula $C_{52}H_{62}O_{20}N_4$ (fumarate salt).

O,O′-*glutaryl (dibenzyl) bispilocarpate fumarate (compound 2)*

A diester was prepared from the pilocarpic acid benzyl monoester (633 mg, 2.00 mmol) with glutaryl chloride (135 mg, 0.80 mmol) by the procedure described above to give 477 mg (0.65 mmol) of free base of *O,O′*-glutaryl (dibenzyl) bispilocarpate. The compound formed a salt with three equivalents of fumaric acid. The diester fumarate was crystallized from toluene/petroleum ether to give 478 mg (0.44 mmol) of bispilocarpic acid diester fumarate. The yield was 55%, m.p. 55–58°C, $\phi_d^{20} = 1.5330$ (free base). Formula $C_{53}H_{64}O_{20}N_4$ (fumarate salt).

O,O′-*adipoyl (dibenzyl) bispilocarpate fumarate (compound 3)*

A diester was prepared from the pilocarpic acid benzyl monoester (406 mg, 1.28 mmol) with adipoyl chloride (108 mg, 0.59 mmol) by the procedure described earlier to give 324 mg (0.44 mmol) of free base of *O,O′*-adipoyl (dibenzyl) bispilocarpate. The compound formed a salt with three equivalents of fumaric acid. The diester fumarate was crystallized from toluene/petroleum ether to give 327 mg (0.30 mmol) of bispilocarpic acid diester fumarate. The yield was 68%, m.p. 98–100°C,



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1. $-\overset{\alpha}{CH_2}\overset{\alpha}{CH_2}-$

2. $-\overset{\alpha}{CH_2}\overset{\beta}{CH_2}\overset{\alpha}{CH_2}-$

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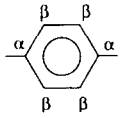
5. 

Figure 1
Structures of *O,O'*-dicarboxylate (dibenzyl) bispilocarpates, i.e. bispilocarpic acid diesters studied.

ϕ_d^{20} = 1.5340 (free base). Formula $C_{54}H_{66}O_{20}N_4$ (fumarate salt).

O,O'-fumaryl (dibenzyl) bispilocarpate fumarate (compound 4)

A diester was prepared from the pilocarpic acid benzyl monoester (633 mg, 2.00 mmol) with fumaryl chloride (123 mg, 0.80 mmol) by the procedure described earlier giving 159 mg (0.22 mmol) of free base of *O,O'*-fumaryl (dibenzyl) bispilocarpate. The compound formed a salt with three equivalents of fumaric acid. The diester fumarate was crystallized from toluene/petroleum ether to give 67 mg (0.06 mmol) of bispilocarpic acid diester fumarate. The yield was 6%, m.p. 64–66°C, ϕ_d^{20} = 1.5415 (free base). Formula $C_{52}H_{60}O_{20}N_4$ (fumarate salt).

O,O'-terephthaloyl (dibenzyl) bispilocarpate fumarate (compound 5)

A diester was prepared from the pilocarpic acid benzyl monoester (633 mg, 2.00 mmol) with terephthaloyl chloride (162 mg, 0.80 mmol) by the procedure described earlier to give 488 mg (0.64 mmol) of free base of *O,O'*-terephthaloyl (dibenzyl) bispilocarpate. The compound formed a salt with three equivalents of fumaric acid. The diester fumarate was crystallized from toluene/petroleum ether to give 370 mg (0.33 mmol) of bispilocarpic acid diester fumarate. The yield was 42%, m.p. 130–133°C, ϕ_d^{20} = 1.5510 (free base). Formula $C_{56}H_{62}O_{20}N_4$ (fumarate salt).

Melting point and index of refraction

The uncorrected melting points of bispilocarpic acid diesters as fumarate salts were determined using a Reichert Thermovar apparatus (Vienna, Austria). Index of refraction of bispilocarpic acid diesters as free bases were measured with an Atago Illuminator refractometer (Japan) at room temperature.

Liquid chromatography

Liquid chromatography (LC) was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman variable UV-detector 166 (set at 215 nm), System Gold data module (Beckman, San Ramon, USA), Marathon autosampler equipped with column thermostat (Spark Holland, AJ Emmen, The Netherlands) and a 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, USA) was used and the isocratic solvent system was 0.02 M KH_2PO_4 (pH 4.5)–methanol (29:71, v/v). The flow rate was 1.0 ml min^{-1}.

Thermospray liquid chromatography–mass spectrometry

The solvents were delivered by a Beckman model 112 pump and samples were injected with a Rheodyne 7125 injector (loop volume 20 μl). The compounds were separated on a deactivated Supelcosil LC8-DB reversed-phase column (15 cm × 4.6 mm i.d., 5 μm). The isocratic solvent system was 0.2 M ammonium acetate–acetonitrile (32:68, v/v) and flow-rate was 1.0 ml min^{-1}.

A VG thermospray–plasmaspray probe was coupled to a VG Trio-2 quadrupole mass spectrometer (VG MassLab., Manchester, UK). The instrument was operated in the thermospray ionization mode. The thermo-

spray probe temperature was 210°C, the ion source temperature was 150°C and the repeller voltage was 210 V. The other ion source conditions were optimized daily.

Electron impact ionization mass spectrometry

The E.I. mass spectra of the compounds were recorded on a VG 70-250SE magnetic sector mass spectrometer (VG Analytical, Manchester, UK). The resolution of the instrument was adjusted to 10,000. The electron energy was 70 eV, ionization current 500 μA and the ion source temperature was 150°C. Samples were introduced to the mass spectrometer in a glass sample holder with a direct insertion probe. The probe temperature was raised from 30 to 500°C in 2–5 min.

The accurate mass measurement of the molecular ions was carried out automatically with the data system. Perfluorokerosene was used as the reference compound.

NMR-spectroscopy

^{1}H and ^{12}C NMR 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 the ^{1}H nuclei and 62.9 MHz for the ^{13}C nuclei. For ^{1}H measurements, 20–40 mg of sample and for ^{13}C measurements, 40–80 mg of sample was added to 0.6 ml of CD_3OD with Me_4Si (0.1%) as an internal standard. The number of data points in the ^{1}H experiment was 32 kW, total relaxation time 16 s, number of scans 64 and pulse angle 45°. Decoupled ^{13}C NMR spectra were measured using composite pulse sequence with 64 kW data points, 10 s relaxation time and 90° pulse angle.

Results and Discussion

Synthesis of prodrugs of pilocarpine

The popularity of esters as prodrugs is due to the fact that the human organism has plenty of esterases which can hydrolyse prodrug esters to the active parent drug. Pilocarpine, a lactone, has no free hydroxyl or carboxyl functions but Bundgaard *et al.* [19] reported that pilocarpic acid is formed after a base hydrolysis. These authors also found that pilocarpic acid monoesters, prepared from pilocarpic acid, are capable of undergoing a fast and quantitative cyclization to the active pilocarpine under physiological conditions. In this work, a pilocarpic acid monoester has been used to prepare a double prodrug by coupling two pilocarpic acid benzyl monoesters together with spacer chain in order to overcome the lability of monoesters [19]. In this way the amount of pro-moiety per pilocarpine molecule was minimized. *O,O'*-dicarboxylate (dibenzyl) bispilocarpate, i.e. bispilocarpic acid diester, with optimal physicochemical properties should penetrate into the corneal epithelium, hydrolyse there enzymatically to pilocarpic acid monoester (less lipophilic than diester), which penetrates to the stroma and aqueous humour where it cyclizes spontaneously to pilocarpine.

The yield of pilocarpic acid benzyl monoester was modest (35–50%), probably due to the formation of the quaternary derivative of sodium pilocarpate during the alkylation. The quaternary derivative remains in the aqueous dimethylsulphoxide phase during the isolation procedure.

The synthesis of bispilocarpic acid diesters gave yields from 6 to 69%, being typically about 50%. All the compounds formed salts with three equivalents of fumaric acid. Pilocarpic acid diesters have been reported to form salts with 1.5 equivalents of fumaric acid [21, 22] and hence it was anticipated that bispilocarpic acid diesters would also form salts with three equivalents of fumaric acid. The estimated (NMR, LC–UV, TSP-LC–MS) purity of the synthetic products varied from 90 to 98% (w/w) and typical impurities were solvents used in the synthesis procedures, pilocarpine, spacer reagents, and uncompletely esterified reaction products. During the synthesis *O,O'*-glutaryl (dibenzyl) bispilocarpate was found to epimerize. The product obtained contained about 20% of the iso-epimer but was used at such purity in tests of physicochemical properties and kinetics [23].

Analysis of double prodrugs by LC–UV and TSP-LC–MS

The LC–UV procedure was developed in order to provide a suitable method for the routine evaluation of the purity of the synthetic products and for the determination of physicochemical properties of the bispilocarpic acid diesters. A fast reversed-phase LC–UV separation for bispilocarpic acid diesters and synthetic impurities (pilocarpic acid benzyl monoester, pilocarpine and solvents used in synthesis) were established on Supelcosil LC8-DB column with 71% methanol in 20 mM phosphate buffer (pH 4.5) as the mobile phase.

The method produces very narrow and symmetric peaks even without triethylamine in mobile phase. A drawback of the method was the short lifetime of the column. Figure 2 shows a typical LC–UV chromatogram from the synthetic product *O,O'*-adipoyl (dibenzyl) bispilocarpate fumarate.

TSP-LC–MS was applied to obtain structural information, to evaluate the purity of the synthetic products and to identify the im-

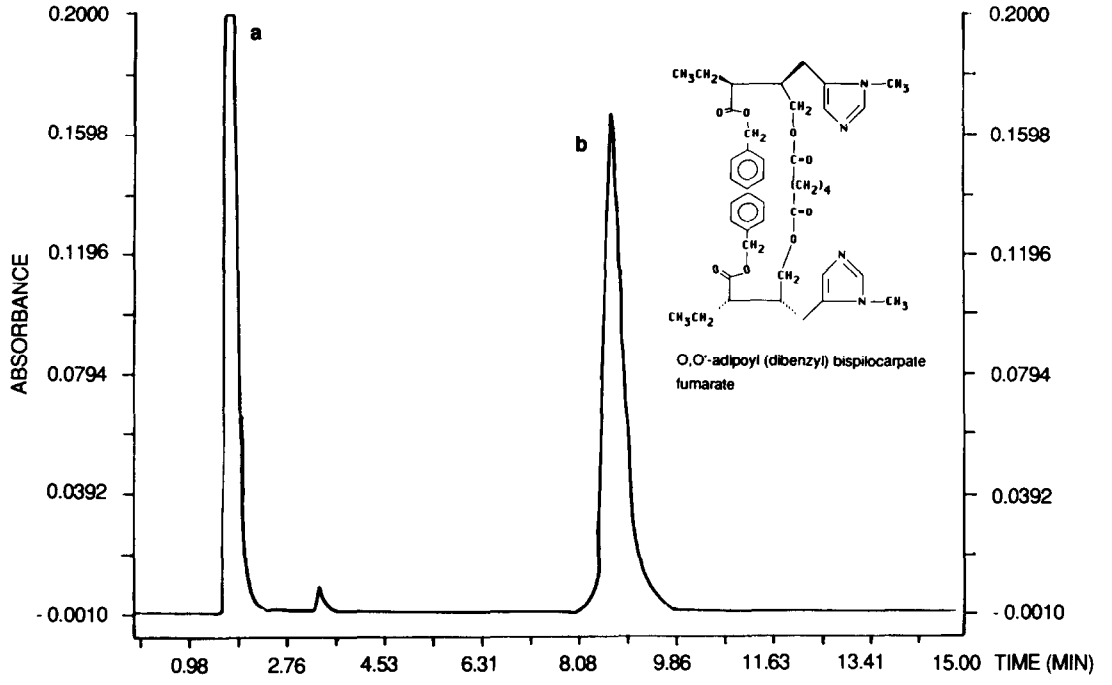


Figure 2
LC–UV chromatogram of the synthetic product of *O,O'*-adipoyl (dibenzyl) bispilocarpate fumarate. Peak a, fumaric acid; peak b, *O,O'*-adipoyl (dibenzyl) bispilocarpate as a free base. LC conditions described in the text.

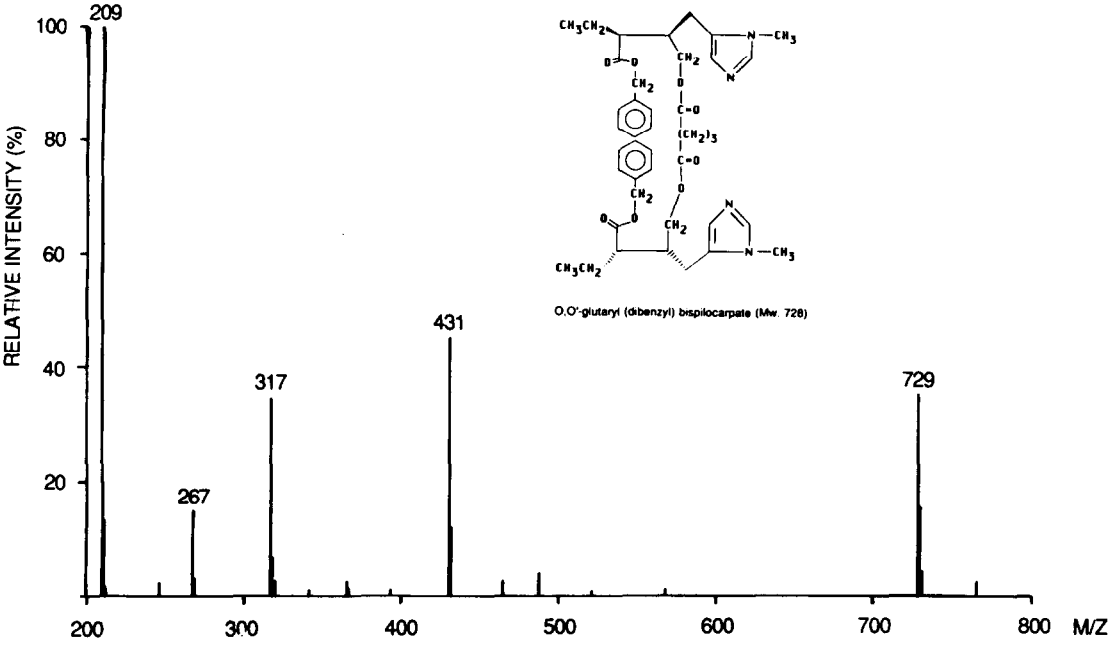


Figure 3
Thermospray mass spectrum of the *O,O'*-glutaryl (dibenzyl) bispilocarpate. The spectrum was obtained by injection 5 μg of the compound via column.

purities of the synthetic products. One limitation of TSP-LC–MS is the need for a volatile buffer in the eluent in order to provide a soft ionization process [24]. Consequently, phosphate buffer was changed to ammonium acetate where ammonium acts as the protonating ion.

Figure 3 shows a typical TSP-mass spectrum obtained after LC-separation of *O,O'*-glutaryl (dibenzyl) bispilocarpate fumarate. The structures of the most important fragment ions are shown in Fig. 4. The protonated molecular ion $[M + H]^+$ or the fragment ion *m/z* 209 [pilocarpine + H] was the base peak in every spectra of the bispilocarpic acid diesters. The most significant peaks and corresponding abundances for each of bispilocarpic acid diesters studied are summarized in Table 1.

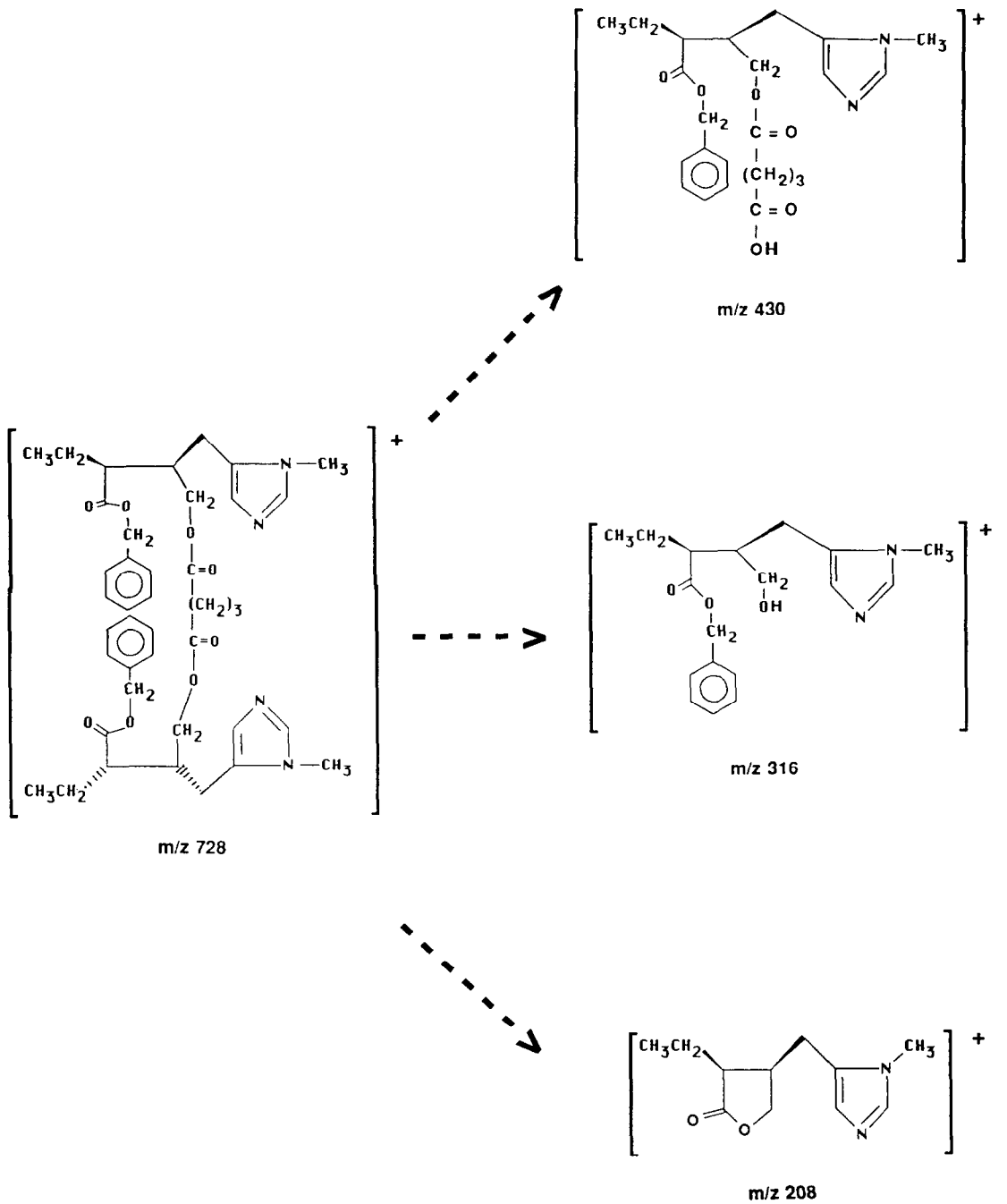


Figure 4
Presumed structures of the fragment ions in thermospray mass spectrum of *O,O'*-glutaryl (dibenzyl) bispilocarpate (less added proton).

Table 1
Relative abundance of the ions in the TSP spectra of *O,O'*-dicarboxylate (dibenzyl) bispilocarpates *m/e*. Numbers in brackets are percentage relative intensity

Compound	$[M + H]^+$	Other ions					
1	715 (47)	751 (10)	417 (32)	317 (57)	267 (15)	209 (100)	
2	729 (36)	766 (3)	431 (45)	317 (35)	267 (15)	209 (100)	
3	743 (100)	780 (75)	765 (28)	480 (8)	445 (10)	317 (23)	209 (26)
4	713 (100)	750 (40)	733 (20)	450 (15)	413 (32)	317 (96)	209 (42)
5	763 (82)	465 (52)	317 (97)	267 (23)	209 (100)		

TSP-LC–MS offers possibilities to analyse samples that need chromatographic separation and that are either difficult or impossible to analyse by gas chromatography–mass spectrometry (GC–MS). TSP-LC–MS has been criticised for its reduced degree of fragmentation which provides less data to identify unknown compounds. However, the TSP-LC–MS system was used here mainly to confirm the presence of compounds known to be present. There were more fragmentation in TSP-spectra of bispilocarpic acid diesters than in typical TSP-spectra.

E.I. mass spectrometry

The bispilocarpic acid diesters were also identified by recording the E.I. mass spectra; accurate molecular weights were established by high-resolution MS (HR-MS) in order to determine the elemental composition of the compounds (in base form).

The E.I. mass spectrum of the *O,O'*-glutaryl (dibenzyl) bispilocarpate exhibits a very low intensity molecular ion M^+ at *m/z* 728 (Fig. 5). There are only a few major fragments in the spectrum due to the labile nature of the compound, a result also seen in corresponding TSP-spectrum. The main fragment ions *m/z* 207, *m/z* 95 and *m/z* 91 result from degradation of the molecular ion to [pilocarpine − H] (*m/z* 207) and from a fragmentation of pilocarpine which involves cleavage of the imidazole ring to give ion at *m/z* 95. The fragment of *m/z* 91 is postulated to be the benzyl ion. Table 2 lists the principal ions with relative abundance of the bispilocarpic acid diesters studied.

The accurate mass values of the compounds are given in Table 3 with corresponding elemental compositions and the error. The error between observed and calculated mass was below 6.5 mmu for all the compounds studied which makes the method reliable for determination of the elemental composition of the bispilocarpic acid diesters.

NMR-spectroscopy

NMR inquiry together with mass spectrometry ensures correct identification and documentation of drug substances. The proton NMR data of the bispilocarpic acid diesters are listed in Table 4. Assignment of the ^1H signals was based on a previously reported ^1H-^1H-correlated COSY experiment for monopilocarpic acid diesters. This could be done because of similarity of the NMR features of both compound groups [22].

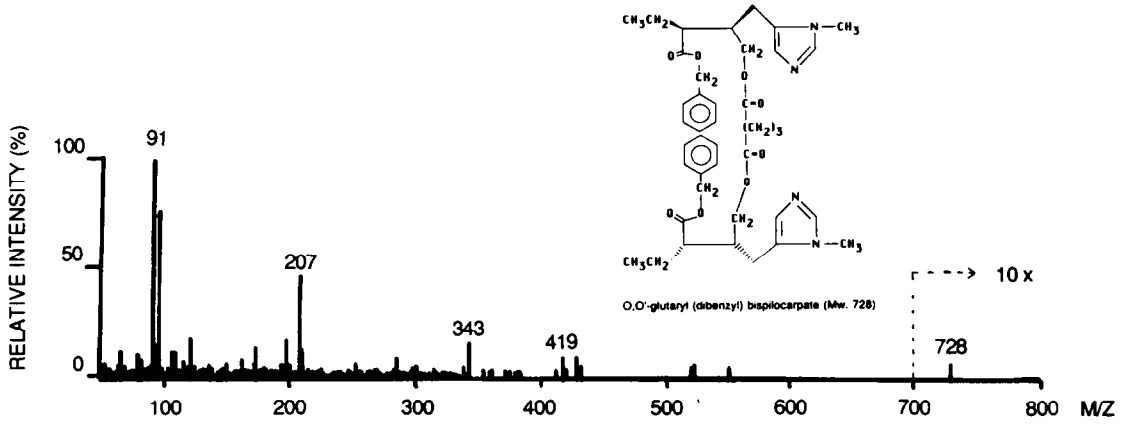


Figure 5
Electron impact ionization mass spectrum (70 eV) of *O,O'*-glutaryl (dibenzyl) bispilocarpate.

Table 2
Relative abundance of the ions in the E.I. spectra of *O,O'*-dicarboxylate (dibenzyl) bispilocarpates *m/e*. Numbers in brackets are percentage relative intensity

Compound	$[M^+]$	Other fragment ions						
1	714 (1)	537 (10)	415 (9)	329 (11)	208 (10)	207 (31)	96 (23)	95 (94)
		91 (100)						
2	728 (0.15)	419 (8)	343 (16)	209 (12)	208 (14)	207 (45)	96 (31)	95 (76)
		91 (100)						
3	742 (5)	741 (8)	629 (39)	625 (52)	536 (75)	535 (100)	459 (69)	445 (43)
		357 (33)	299 (45)	209 (55)	207 (64)	95 (69)	91 (56)	
4	712 (0.4)	411 (41)	407 (34)	318 (56)	317 (96)	299 (39)	209 (67)	207 (40)
		96 (47)	95 (100)	91 (62)				
5	762 (1)	208 (13)	207 (46)	96 (36)	95 (100)	91 (70)		

Table 3
Measured and calculated accurate mass of *O,O'*-dicarboxylate (dibenzyl) bispilocarpates

Compound	Observed mass	Calculated mass	Error (mmu)	Elemental composition (free base)
1	714.36690	714.36285	4.0	$C_{40}H_{50}O_8N_4$
2	728.37347	728.37850	5.0	$C_{41}H_{52}O_8N_4$
3	742.39038	742.39415	3.8	$C_{42}H_{54}O_8N_4$
4	712.34622	712.34720	1.0	$C_{40}H_{48}O_8N_4$
5	762.35638	762.36286	6.5	$C_{44}H_{50}O_8N_4$

Table 4
Proton chemical shifts for compounds 1–5 (ppm) in CD_3OD

	Compounds (as fumarates)				
	1	2	3	4	5
Proton					
N-Me	3.65	3.67	3.65	3.67	3.70
2	8.44	8.47	8.36	8.42	8.46
4	7.17	7.20	7.14	7.18	7.24
6	2.68	2.68	2.68	2.72	2.83
7	2.32	2.32	2.34	2.45	2.54
8	2.53	2.54	2.54	2.57	2.66
9	1.69	1.69	1.69	1.71	1.75
10	0.86	0.87	0.87	0.89	0.91
11	4.09	4.07	4.07	4.22	4.37
12	5.14	5.14	5.14	5.12	5.10
14	7.35	7.35	7.35	7.34	7.32
15	7.35	7.35	7.35	7.34	7.32
16	7.35	7.35	7.35	7.34	7.32
R_α	2.58	2.34	2.30	6.76	—
R_β	—	1.84	1.59	—	8.05
Fum.	6.71	6.71	6.72	6.71	6.72

The limit of detection of impurities and solvent residues was lower than 0.5 mole%. The degree of dimerization was investigated comparing integrals from -N-Me, Ph-CH_2OOC and -CH_2COOR groups. Other impurities were identified and quantitated using signals from the methyl triplet (CH_3CH_2-) of the ethyl side chain and signals from the heterocyclic ring. No significant degradation procedures were observed during NMR measurements.

The formation of epimeric products was monitored by ^{13}C NMR spectroscopy because spectra of diastereoisomeric pairs displayed distinct epimeric signals. From such evidence only the *O,O'*-glutaryl (dibenzyl) bispilocarpate was found to epimerize to the diastereomeric derivative.

Conclusions

Coupling two pilocarpic acid benzyl monoesters with a spacer chain is one way of attempting to overcome the lability of pilocarpic acid benzyl monoester. This type of double-prodrug of pilocarpine also minimizes the amount of the pro-moiety per mole of active pilocarpine. The described LC–UV, TSP-LC–MS, HR-MS, and NMR spectroscopy methods have been applied successfully to the analysis of the *O,O'*-dicarboxylate (dibenzyl) bispilocarpates, i.e. bispilocarpic acid diesters, studied. Studies are in progress to examine the physicochemical and hydrolysis characteristics of these prodrug derivatives.

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