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Evaluation of the antibacterial efficacy of diesters of azelaic acid

Colin Charnock [a,*], Bjarne Brudeli [b], Jo Klaveness [b,c]

[a] *Faculty of Health Sciences, Oslo University College, Postboks 4, St. Olav pl., 0130 Oslo, Norway*
[b] *Drug Discovery Laboratory AS, Oslo Research Park, Gaustadalleen 21, 0349 Oslo, Norway*
[c] *Department of Medicinal Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1155, Blindern, N-0317 Oslo, Norway*

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Abstract

A number of diesters of the topical dermatosis treatment azelaic (nonanedioic) acid were prepared and tested for antibacterial effect. Two esters, bis-[(hexanoyloxy)methyl] nonanedioate and especially bis-[(butanoyloxy)methyl] nonanedioate showed promising activity against acne related bacteria in vitro. No activity of azelaic acid was detected in Mueller Hinton II agar at pH 7.3 when using the agar diffusion method, whereas both esters gave zones of growth inhibition. At pH 5.6, activity of azelaic acid was detected. At this pH, the zones of inhibition and MIC values obtained with azelaic acid were smaller than those of bis-[(butanoyloxy)methyl] nonanedioate for all test organisms. A preparation for topical use containing 20% (w/w) bis-[(butanoyloxy)methyl] nonanedioate, and the commercially available Skinoren® (20% (w/w) azelaic acid), were compared for antibacterial effect against cutaneous bacteria using contact plate analyses of the skin. Though Skinoren® was usually most effective, the differences were not statistically significant. Furthermore, bacteria surviving contact with the topical preparations were invariably more sensitive to the ester than to azelaic acid upon subculturing onto agar (pH 5.6) containing either preparation at 0.2–0.7 mg/ml. This might indicate that other factors, such as the composition of the cream base, mitigate the antibacterial activity of the ester. It is proposed that the pharmacological and microbiological properties of bis-[(butanoyloxy)methyl] nonanedioate are worthy of further study based on an extended screening of acne sufferers.

Keywords: Azelaic acid; Diester; Antibacterial efficacy

1. Introduction

Azelaic acid is widely used as a therapeutic agent in dermatology. Among its uses are the treatment of acne (Hjorth and Graupe, 1989; Fitton and Goa, 1992), where it has been shown to be bactericidal for *Propionibacterium acnes* and a number of other cutaneous microorganisms (Leeming et al., 1986; Bojar et al., 1991). *P. acnes* and *Staphylococcus epidermidis* are the predominant microorganisms on acne effected skin (Bojar et al., 1994). *P. acnes* is considered to be one of the factors involved in the development of acne (Holland, 1989). This species appears to initiate the inflammatory process by producing neutrophil chemotactic factors. Once neutrophils attracted by bacterial chemoattractants reach the inflamed site, they release inflammatory mediators such as lysosomal enzyme and reactive oxygen species (Jain et al., 2002).

The mechanism(s) of the reported bactericidal activity of azelaic acid remains to be confirmed. However, effects on enzyme activities, macromolecular synthesis, and intracellular pH have been suggested (Passi et al., 1984; Hu et al., 1986; Bojar et al., 1988, 1991, 1994). Non-viable cells of *P. acnes* do not accumulate azelaic acid and an energized cell membrane appears to be necessary for its uptake in the bacterial cell (Bojar et al., 1988, 1991). The pH of the growth medium is probably the single most important factor governing the sensitivity of microorganisms to azelaic acid (Bojar et al., 1991).

It has been suggested that topical antimicrobials, and antibiotics in particular, should be used less often than in the past, and only for short periods in order to avoid the development of resistances (Gollnick and Krautheim, 2003). Due to emerging *P. acnes* resistance to tetracycline and erythromycin, the use of antibiotics in acne therapy should be minimized (Eady et al., 2003). No bacterial resistances to

* Corresponding author. Present address: Høgskolen i Oslo, Reseptarutdanningen, Pilestredet 52, 0167 Oslo, Norway. Tel.: +47-22-452348; fax: +47-22-452335.
E-mail address: colin.charnock@hf.hio.no (C. Charnock).

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azelaic acid have as yet been reported, and the compound is none toxic (Mingrone et al., 1983). Thus alone or in combination with other agents, azelaic acid is a valuable alternative in acne treatment (Gollnick and Krautheim, 2003).

Prodrugs derived by for example esterification of the carboxyl moiety of organic acids (such as penicillin), form the basis for several antibacterial treatments. Esterification imparts new pharmacological properties, including increased lipophilicity. The esterification of azelaic acid may aid in its penetration of serbaceous skin exudates as well as the cell membrane of infecting microbes. Upon or during entry into the bacterial cell the free acid, it is proposed, will be released through the activity of esterases. Acyloxymethyl esters are known to be good substrates for esterases (Jansen and Russell, 1965). Within the field of antibiotics, such diesters have been used to improve the bioavailability of penicillins (for example, pivampicillin versus ampicillin). With this in mind, a series of diesters of azelaic acid were produced and screened for antibacterial activity.

2. Materials and methods

The synthesized products were characterized by NMR and the purity was determined by GC. The ^{1}H NMR spectra were recorded on a Bruker Spectrospin Avance 200 and 300 MHz spectrometer. The ^{13}C NMR spectra were recorded at 75 or 50 MHz. Chemical shifts are reported in ppm using $CDCl_3$ (77.00 ppm) as reference in ^{13}C spectra, and residual $CHCl_3$ (7.24 ppm) in ^{1}H spectra.

The electrospray (ES) mass spectra were recorded on a VG Quattro II instrument (Micromass Ltd., Altrincham, England). The flow rate was 0.1 ml/min (acetonitrile/H_2O, 1:1). The spectra are presented as *m*/*z*.

GC analyses were carried out on a Shimadzu GC-14A gas chromatograph equipped with a flame-ionization detector and a 30 m × 0.25 μm i.d. glass capillary column coated with poly(dimethylsiloxane), at a flow rate of nitrogen of 40 ml/min. The column temperature ranged from 80 to 200 °C at 8 °C/min. The temperatures of the injector and detector were 250 °C.

N,*N*-Dimethylformamide (Sigma–Aldrich, St. Louis, MO, USA), and diethyl ether (Riedel-de Haen, Seelze, Germany) were both of analytical grade. Azelaic acid monomethyl ester (Aldrich) was of technical grade (85%) and used without any purification. All other chemicals were of analytical or reagent grade and were obtained from commercial sources.

3. Synthesis

3.1. Synthesis of chloromethyl esters (*1*–*4*)

Chloromethyl pivalate and 1-chloroethyl ethyl carbonate (respectively Cl–CH_2–O–CO–*t*–Bu and Cl–CH(CH_3)–O–CO–O–Et) are commercially available and were purchased (Aldrich).

The other chloromethyl esters were prepared using a slight modification of a method described in the literature (Ulich and Adams, 1921). Acid chlorides (0.10 mole scale) were mixed in equimolecular amounts with paraformaldehyde in a round bottom flask and heated at 80 °C for 2 h, then at 120 °C until the latter disappeared. The reaction mixture was distilled in vacuo and the chloromethyl esters were obtained as colourless liquids.

3.1.1. Synthesis of chloromethyl benzoate (*1*)

Colourless oil (bp 116–117 °C, 10 mbar). Yield: 7.52 g (44.0%). ^{1}H NMR (200 MHz): δ 8.18–7.46 (m, 5H), 6.00 (s, 2H). ^{13}C NMR (50 MHz): δ 133.9, 131.4, 130.0, 128.9, 128.5, 69.2.

3.1.2. Synthesis of chloromethyl butyrate (*2*)

Colourless oil (bp 148–149 °C). Yield: 6.89 g (50.4%). ^{1}H NMR (300 MHz): δ 5.72 (s, 2H), 2.39 (t, *J* 7 Hz, 2H), 1.76–1.65 (m, *J* 7 Hz, 2H), 0.97 (t, *J* 7 Hz, 3H). ^{13}C NMR (75 MHz): 171.5, 68.6, 31.0, 24.1, 13.4.

3.1.3. Synthesis of chloromethyl hexanoate (*3*)

Colourless oil (bp 118–119 °C, 25 mbar). Yield: 6.54 g (39.7%). ^{1}H NMR (300 MHz): δ 5.66 (s, 2H), 2.33 (t, *J* 7 Hz, 2H), 1.65–1.58 (m, *J* 7 Hz, 2H), 1.30–1.26 (m, 4H), 0.86 (t, *J* 7 Hz, 3H). ^{13}C NMR (75 MHz): δ 171.6, 68.4, 33.8, 31.0, 24.1, 22.1, 13.7.

3.1.4. Synthesis of chloromethyl octanoate (*4*)

Colourless oil (bp 140–141 °C, 25 mbar). Yield: 9.11 g (47.3%). ^{1}H NMR (300 MHz): δ 5.67 (s, 2H), 2.34 (t, *J* 7 Hz, 2H), 1.64–1.58 (m, 2H), 1.30–1.22 (m, 8H), 0.84 (t, *J* 7 Hz, 3H). ^{13}C NMR (75 MHz): δ 171.7, 68.5, 33.9, 31.5, 28.9, 28.8, 24.5, 22.5, 13.9.

3.2. Synthesis of diesters of dodecanedioic acid and azelaic (nonanedioic) acid (*5*–*14*)

Azelaic (nonanedioic) acid (**a**), dodecanedioic acid (**b**) and azelaic acid monomethyl ester (**c**) were reacted with cesium carbonate to their corresponding cesium salts. Subsequent addition of different chloroalkyl esters gave the corresponding diesters of the respective aliphatic acids.

A suspension of Cs_2CO_3 (10 mmol for monoacid; 20 mmol for diacids), NaI (trace amounts) and azelaic acid/dodecanedioic acid/azelaic acid monomethyl ester (0.010 mol scale) in DMF (40 ml) was stirred for 30 min at 60 °C. The chloroalkylester (10 mmol for monoacid; 20 mmol for diacids) was added dropwise to the solution and stirred at 60 °C for 48 h. The reaction mixture was thereafter cooled to ambient temperature, evaporated in vacuo and diethyl ether (40 ml) was added to the residue. The organic layer was washed with saturated $NaHCO_3$

(3 × 20 ml), filtered, dried with $MgSO_4$ and evaporated in vacuo to give the diesters.

*3.2.1. Synthesis of bis-[(benzoyloxy)methyl] nonanedioate (**5**)*

White waxy solid. Yield: 3.52 g (77.2%). ^{1}H NMR (300 MHz): δ 8.08 (d, 4H), 7.56 (t, 2H), 7.42 (t, 4H), 5.97 (s, 4H), 2.32 (t, J 7 Hz, 4H), 1.61–1.56 (m, J 7 Hz, 4H), 1.28–1.25 (s, 6H). ^{13}C NMR (75 MHz): δ 172.3, 165.2, 133.6, 130.0, 128.9, 128.4, 79.7, 33.8, 28.7, 28.6, 24.4. MS (ES): 479.2 $[M + Na]^+$.

*3.2.2. Synthesis of bis-[(butanoyloxy)methyl] nonanedioate (**6**)*

Colourless oil. Yield: 0.76 g (49.0%). ^{1}H NMR (300 MHz): δ 5.70 (s, 4H), 2.29 (t, 8H), 1.65–1.55 (m, 8H), 1.27 (s, 6H), 0.90 (t, J 7 Hz, 6H). ^{13}C NMR (75 MHz): δ 172.3, 162.5, 78.9, 35.7, 33.8, 28.6, 24.4, 18.0, 13.4. MS (ES): 411.2 $[M + Na]^+$.

*3.2.3. Synthesis of bis-[(2,2-dimethylpropanoyloxy)methyl] nonanedioate (**7**)*

Colourless oil. Yield: 4.77 g (57.3%). ^{1}H NMR (300 MHz): 5.68 (s, 4H), 2.27 (t, J 7 Hz, 4H), 1.57–1.53 (m, 4H), 1.26–122 (s, 6H), 1.14–1.10 (m, 18H). ^{13}C NMR (75 MHz): 172.2, 164.8, 79.2, 38.7, 33.9, 29.0, 28.8, 26.8, 24.5. MS (ES): 439.3 $[M + Na]^+$.

*3.2.4. Synthesis of (2,2-dimethylpropanoyloxy)methyl methyl nonanedioate (**8**)*

Colourless oil. Yield: 2.13 g (67.5%). ^{1}H NMR (200 MHz): δ 5.74 (s, 2H), 3.66 (s, 3H), 2.38–2.26 (q, J 7 Hz, 4H), 1.63–1.56 (m, J 7 Hz, 4H), 1.31 (s, 6H), 1.21 (s, 9H). ^{13}C NMR (50 MHz): δ 177.0, 174.0, 172.2, 79.1, 51.3, 38.6, 33.9. 33.8, 28.7, 28.7, 28.6, 26.7, 24.7, 24.4. MS (ES): 339.3 $[M + Na]^+$.

*3.2.5. Synthesis of bis-[1-(ethoxycarbonyloxy)ethyl] nonanedioate (**9**)*

Yellow oil. Yield: 1.92 g (45.6%). ^{1}H NMR (200 MHz): δ 6.81–6.73 (q, J 5.8 Hz, 2H), 4.30–4.11 (q, J 7 Hz, 4H), 2.33 (t, J 7 Hz, 4H), 1.55–1.48 (m, 4H), 1.46 (d, J = 5.8 Hz, 2H), 1.32–1.18 (m, 12H). ^{13}C NMR (50 MHz):178.3, 171.6, 152.9, 91.0, 64.8, 33.9, 28.7, 24.5, 19.4, 14.0. MS (ES): 443.2 $[M + Na]^+$.

*3.2.6. Synthesis of 1-(ethoxycarbonyloxy)ethyl methyl nonanedioate (**10**)*

Colourless oil. Yield: 2.40 g (75.1%). ^{1}H NMR (200 MHz): δ 6.78–6.70 (q, J 5.8 Hz, 1H), 4.20 (q, J 7 Hz, 2H), 3.64 (s, 3H), 2.34–2.24 (m, 4H), 1.63–1.53 (m, 4H), 1.49 (d, J 5.8 Hz, 2H), 1.33–1.24 (m, 9H). ^{13}C NMR (50 MHz): δ 174.5, 172.0, 162.9, 153.3, 91.4, 64.7, 51.7, 36.8, 34.3, 31.7, 29.2, 25.1, 24.8, 19.0, 14.4. MS (ES): 341.2 $[M + Na]^+$.

*3.2.7. Synthesis of bis-[(hexanoyloxy)methyl] nonanedioate (**11**)*

Colourless oil. Yield: 2.45 g (51.9%). ^{1}H NMR (300 MHz): δ 5.72 (s, 4H), 2.43–2.32 (m, 8H), 1.75–1.64 (m, 8H), 1.36–1.33 (m, 14H), 0.91 (t, J 7 Hz, 6H). ^{13}C NMR (75 MHz): δ 172.8, 162.7, 79.4, 34.4, 34.3, 34.2, 31.5, 29.1, 29.1, 24.8, 24.6, 22.6, 14.2. MS (ES): 495.2 $[M + Na]^+$.

*3.2.8. Synthesis of bis-[(octanoyloxy)methyl] nonanedioate (**12**)*

Colourless oil. Yield: 2.37 g (47.9%). ^{1}H NMR (200 MHz): δ 5.72 (s, 4H), 2.37 (m, 8H), 1.64–1.57 (m, 8H), 1.30 (s, 22H), 0.86 (t, J 7 Hz, 6H). ^{13}C NMR (50 MHz): δ 172.8, 162.3, 79.4, 34.4, 34.3, 34.2, 31.5, 29.1, 29.1, 24.8, 24.6, 22.6, 14.2. MS (ES): 523.3 $[M + Na]^+$.

*3.2.9. Synthesis of bis-[(2,2-dimethylpropanoyloxy)methyl] dodecanedioate (**13**)*

Colourless oil. Yield: 2.56 g (56.0%). ^{1}H NMR (300 MHz): δ 5.96 (s, 4H), 2.48 (t, J 7 Hz, 4H), 1.58–1.52 (m, J 7 Hz, 4H), 1.21–1.15 (m, 30H). ^{13}C NMR (75 MHz): δ 176.3, 172.5, 79.2, 68.7, 38.6, 33.9, 29.2, 28.8, 26.7, 25.5, 24.6. MS (ES): 481.4 $[M + Na]^+$.

*3.2.10. Synthesis of bis-[1-(ethoxycarbonyloxy)ethyl] dodecanedioate (**14**)*

Yellow oil. Yield: 2.19 g (47.5%). ^{1}H NMR (200 MHz): δ 6.79–6.71 (q, J 5.8 Hz, 2H), 4.25–4.10 (q, J 7 Hz, 4H), 2.34 (t, J 7 Hz, 4H), 1.57–1.50 (m, 4H), 1.46 (d, J 5.8 Hz, 2H), 1.32–1.18 (m, 18H). MS (ES): 485.5 $[M + Na]^+$.

3.3. Antibacterial testing

The following bacterial strains were included in the study: *Staphylococcus aureus* (type strain, ATCC 25923), *Staphylococcus epidermidis* (type strain, DSM 20044), *Propionibacterium acnes* (type strain, DSM 1897).

Antibacterial activity was evaluated by means of the agar diffusion method. Bacteria were either spread on the surface of an agar plate or mixed into the agar before pouring. For the surface inoculation technique, a suspension of bacteria in physiological salt water with a density equivalent to McFarland 1.0 was used. When included in the agar, 10 μl of a McFarland 3.0 (or 6 μl of a McFarland 4.0) suspension was used per 100 ml of growth medium. Typically 35 ml of bacteria-inoculated growth medium was poured into an empty petridish (diameter = 140 mm).

When surface inoculation was used, antibacterials were applied to the plate in the form of impregnated paper discs (Oxoid, Basingstoke, UK). Discs were inoculated with liquid esters and allowed to stand for 30 min before being inverted onto the seeded plate, such that the impregnated surface came into contact with the agar. Solid esters and azelaic acid were placed directly onto the agar surface over an area equivalent to a paper disc. An uninoculated disc was then placed onto the test substance. If bacteria were included in

the agar, wells with a bore of 5 mm were punched out of the agar and the antibacterial agent was added to the well.

Plates were incubated at 35 ± 1 °C for all test bacteria. Zones of inhibition were usually measured after 24 h incubation. However, for *P. acnes* which is slow growing incubation periods of up to 10 days were necessary.

The following media were used: Mueller Hinton agar II (M–H; Oxoid) pH 7.3 ± 0.1 and yeast extract glucose agar, YEGA (pH 5.6 ± 0.1) which was made as follows: yeast extract (acumedia, Baltimore, Maryland, US), 5 g; glucose (Norsk Medisinaldepot, Oslo, Norway) 10 g and Bacto agar (Difco, Detroit, Michigan US), 20 g, were dissolved in 600 ml of 0.2 M NaH_2PO_4. Thereafter 0.2 M Na_2HPO_4 was added until the pH was 5.6 ± 0.1. The solution was then made up to 1000 ml with deionised water before sterilising by autoclaving. A similar medium, YEGA 1, for use with *P. acnes* contained 2.5 g yeast extract and 2.5 g of tryptone (Difco) instead of 5 g yeast extract. Additionally, 10 ml of filter sterilised 50× basal freshwater salts (Sigma) per 1000 ml medium was added after autoclaving. Sheeps blood agar was used for susceptibility testing of *P. acnes* at pH 7.4 ± 0.1. An anaerobic environment for the growth of *P. acnes* was generated using the Anaerogen® gas pack system (Oxoid).

MIC values for bis-[(butanoyloxy)methyl] nonanedioate and azelaic acid were estimated at pH 5.6±0.1 using YEGA and YEGA 1. One test involved streaking out a small amount of cellular material onto agar in which the agent was included. The plates were examined for the growth over a period of 10 days, and the growth was graded by comparison with a control plate containing no agent. MICs were also determined by inclusion of bacteria (10^5 ml^{-1} agar) and serial dilutions of antimicrobial agent in YEG/YEGA1 in the wells of a microtiter plate (Nunc, Roskilde, Denmark). After mixing, wells were covered with a sterile membrane (Nunc) which was punctured with sterile needle (except for wells containing *P. acnes*); thereafter the plate lid was replaced. Plates were incubated as described above, and wells were examined visually for growth over a period of 2 weeks. All MIC determinations were repeated at least once with esters from different rounds of production.

Testing of the bacterial efficacy of (**6**) against cutaneous bacteria was performed by mixing the ester at 20% (w/w) in a cream base (Merck, NJ, USA). The commercially available product Skinoren® (20% azelaic acid (w/w)) was tested for comparison. The creams were massaged into the finger pads or faces of the co-workers and allowed to work for variously 5–30 min. The number of surviving bacteria was then determined by taking finger dabs on tryptone soya agar (TSA, Oxoid), or, in the case of the face, by pressing TSA contact plates (diameter = 55 mm) onto the treated area. Similar testing of untreated areas of skin was used for control purposes. Colonies which grew on the plates were subcultured onto YEG containing 0.2–0.7 mg/ml of azelaic acid or (**6**), and examined for growth over a period of 2 weeks.

4. Results and discussion

Chloromethyl esters were obtained as colourless oils in yields ranging from 39.7 to 54% (Fig. 1). Diesters of azelaic (nonanedioic) and dodecanoic acids were obtained as colourless oils or waxy solids, in yields ranging from 45.6 to 77.2% (Fig. 2). According to the GC analyses, the products isolated from the reaction mixtures were almost pure (>95%), and needed no further purification.

An initial screening using the agar diffusion method, showed that 2 of the test esters had good antibacterial activity. These were bis-[(hexanoyloxy)methyl] nonanedioate (**11**), and especially bis-[(butanoyloxy)methyl] nonanedioate (**6**). The other esters gave considerably smaller or no zones of inhibition. Fig. 3 is typical for the results obtained when testing esters and azelaic acid at pH 7.3±0.1 on **M**–H. Both aforementioned esters showed good activity against the type strains of *S. aureus*, *S. epidermidis* and *P. acnes* (see below). No activity of azelaic acid against these species was detected at pH 7.3±0.1. The superior activities of (**6**) and (**11**) compared to other esters tested, may in part be a consequence of their being none-bulky aliphatic molecules. The more bulky bis-[(2,2-dimethylpropanoyloxy)methyl] dodecanedioate and the aromatic groups of bis-[(benzoyloxy)methyl] nonanedioate would, for example, be expected to present greater steric hindrance to their passage across the bacterial cell membrane. Bis-[(butanoyloxy)methyl] nonanedioate (**6**) was chosen for further study.

The antibacterial activities demonstrated for esters could be explained by their being hydrolysed to azelaic acid by esterases during or upon entry into the bacterial cell. There are no published characterizations of intra- or

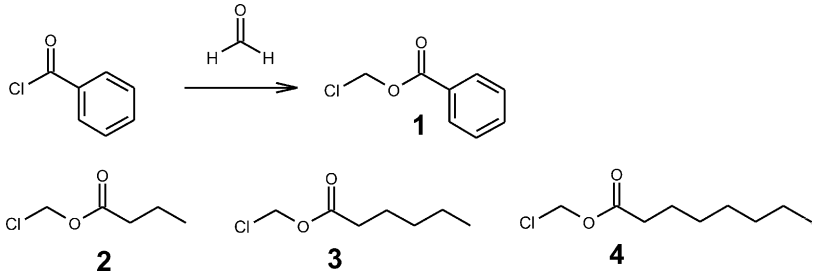

Fig. 1. Chloromethyl esters and their mode of synthesis. Key: 1, chloromethyl benzoate; 2, chloromethyl butyrate; 3, chloromethyl hexanoate; 4, chloromethyl octanoate.

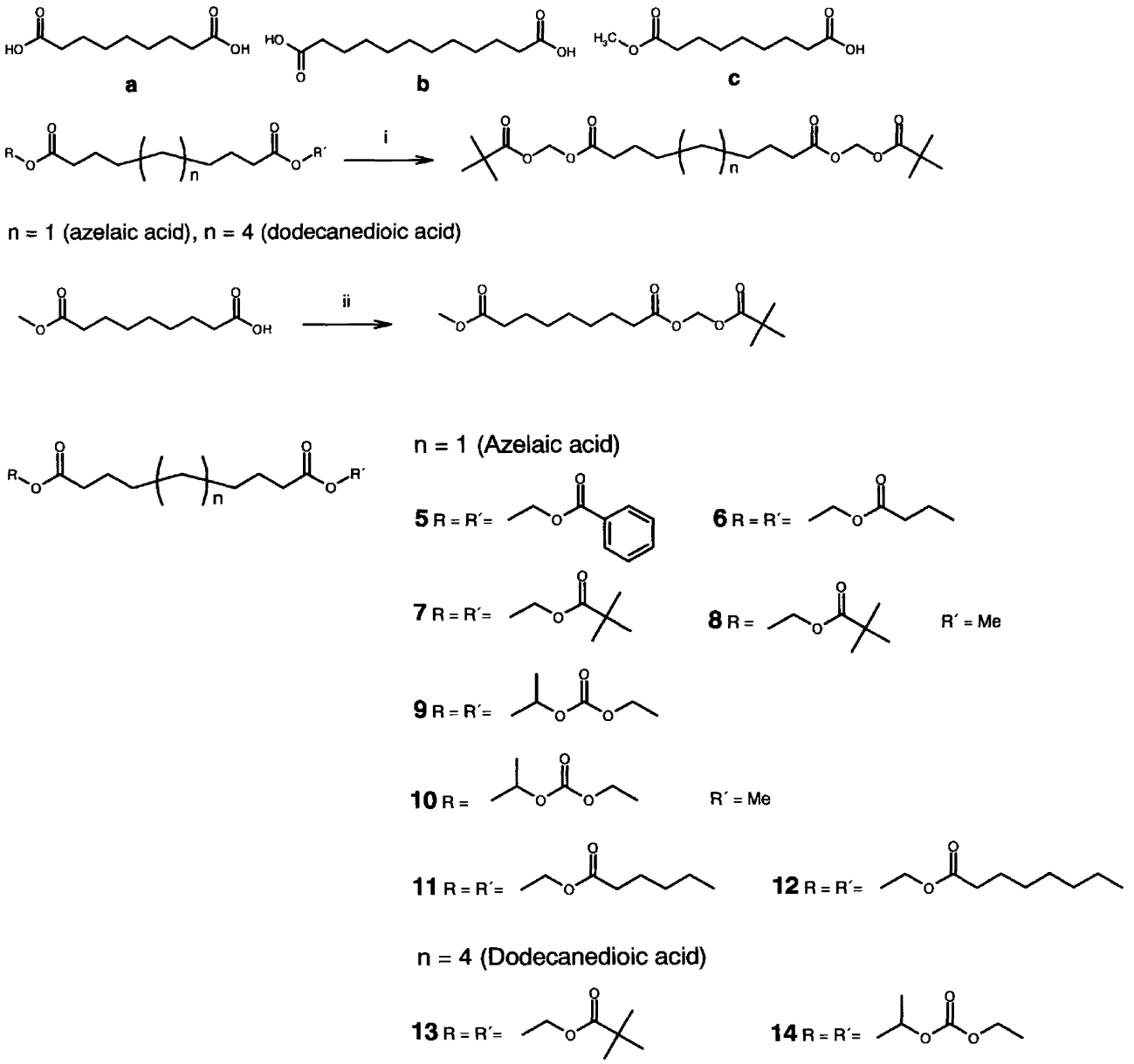

Fig. 2. Diesters of azelaic (nonanedioic) and dodecanedioic esters and their mode of synthesis. Key: **a**, azelaic acid; **b**, dodecanedioic acid; **c**, azelaic acid monomethyl ester; **i–ii**, Cs_2CO_3, chloromethyl pivalate, DMF, 60 °C, 48 h; **5**, bis-[(benzoyloxy)methyl] nonanedioate; **6**, bis-[(butanoyloxy)methyl] nonanedioate; **7**, bis-[(2,2-dimethylpropanoyloxy)methyl] nonanedioate; **8**, (2,2-dimethylpropanoyloxy)methyl methyl nonanedioate; **9**, bis-[1-(ethoxycarbonyloxy)ethyl] nonanedioate; **10**, 1-(ethoxycarbonyloxy)ethyl methyl nonanedioate; **11**, bis-[(hexanoyloxy)methyl] nonanedioate; **12**, bis-[(octanoyloxy)methyl] nonanedioate; **13**, bis-[(2, 2-dimethylpropanoyloxy)methyl] dodecanedioate; **14**, bis-[1-(ethoxycarbonyloxy)ethyl] dodecanedioate.

extracellular hydrolases of esters of azelaic acid. However, carboxylesterases (ECC 3.1.1.1) have been described for a number of bacterial species (see Arpigny and Jaeger, 1999 for overview) and true extracellular lipases (E.C.3.1.1.3) of *S. aureus* (Lee and Iandolo, 1986; Kuroda et al., 2001; Baba et al., 2002), *S. epidermidis* (Farrell et al., 1993; Simons et al., 1998; Longshaw et al., 2000) and *P. acnes* (Ingham et al., 1981; Miskin et al., 1997) have been reported. Lipids are found ubiquitously on the surface of human skin, and are largely composed of sebum-derived triacylglycerols (Nicolaides, 1974). It has been suggested that lipases may be important for the colonization and persistence of resident organisms on the skin, possibly in terms of nutrition or by the release of free fatty acids which may promote adherence to and colonization of sebaceous follicles (Gribbon et al., 1993).

Normal skin pH has been estimated to be 5.0–6.0 (Noble, 1968). A pH of 5.6 was chosen as representative following the lead of others (Bojar et al., 1988, 1991) who have conducted tests similar to those in the present study. At

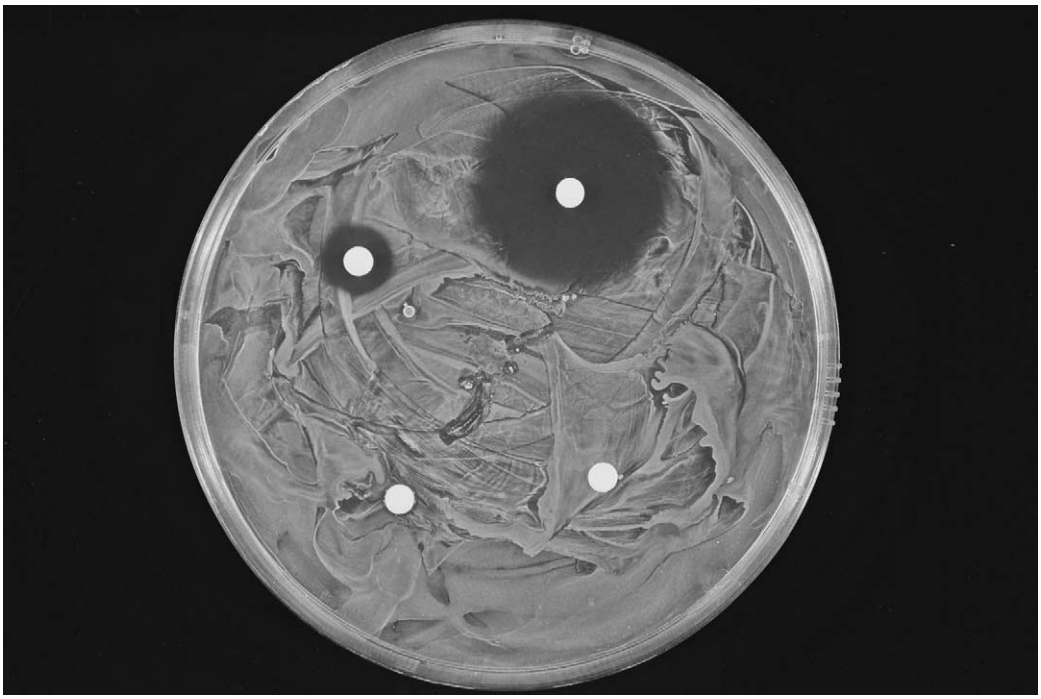

Fig. 3. Inhibition of growth of *S. aureus* by esters of azelaic acid at pH 7.3 on Mueller Hinton II agar using the surface inoculation technique. Key: top left—bis-[1-(ethoxycarbononyloxy)ethyl] nonanedioate (10 mg); top right—bis-[(hexanoyloxy)methyl] nonanedioate (10 mg); bottom left—1-(ethoxycarbonyloxy)ethyl methyl nonanedioate (10 mg); bottom right—azelaic acid (10 mg).

pH 5.6 antibacterial activity of azelaic acid was detected (Nicolaides, 1974; Nicolaides and Wells, 1957). The strong pH dependency of the activity of azelaic acid has been extensively discussed previously, and is probably a consequence of pH-dependent uptake mechanisms (Bojar et al., 1991). The zones of inhibition obtained were smaller than those obtained with (**6**) when used against *S. aureus* and *S. epidermidis*. At pH 5.6, *P. acnes* grew slowly on YEGA 1. After 1 week, zones of inhibition were visible, these were, however, invariably irregular and difficult to standardise. The zones of inhibition of *P. acnes* obtained with azelaic acid and (**6**) appeared to be of approximately equal size. Table 1 summarises the results of sensitivity testing of *S. epidermidis*, *S. aureus* and *P. acnes* using the agar diffusion method. Fig. 4 shows the zones of inhibition of *S. aureus* obtained with azelaic acid and (**6**) at pH 5.6. The agar-diffusion tests were supplemented with an investigation of the bacterial MICs. The $MIC_{azelaic\ acid}$ and $MIC_{(6)}$ were determined at pH 5.6, by scoring the density of growth (0, +, ++, +++) on antibacterial-amended agar with respect to growth on a control plate (+++). The results are summarised in Table 2. The $MIC_{azelaic\ acid}$ was for all species 1.5–4.5 mg/ml. The corresponding value for (**6**) was 0.25–0.75 mg/ml. The MIC-values obtained using the microtiter plate method were 0.90–1.8 mg/ml (azelaic acid) and 0.05–0.15 mg/ml (ester). The results of the MIC testing are thus in general agreement with each other and with the results of susceptibility testing using the agar diffusion method. If the simple in vitro tests described give a good indication of the therapeutic potential of the esters (**6**) may function as well, or better, than azelaic acid in the treatment of dermatitis. Furthermore, any putative condition causing a local increase in skin pH would

Table 1
Diameters of zones of bacterial inhibition obtained using azelaic acid and its ester bis-[(butanoyloxy)methyl] nonanedioate (**6**)

	Diameter of inhibition zone, $\sigma \pm$ S.D. (mm) (amount of antibacterial tested (mg))			
	S. aureus	*S. epidermidis*	*P. acnes*[a]	
pH	5.6 ± 1	5.6 ± 1	5.6 ± 1	7.4 ± 1
Ester	59 ± 8 (10) 79 ± 3 (20)	102 ± 3 (10) 111 ± 16 (20)	43 ± 15 (10)	47 ± 4 (10)
Azelaic acid	36 ± 3 (10) 45 ± 2 (20)	29 ± 6 (10) 36 ± 3 (20)	43 ± 15 (10)	0 (10–20)

Antibacterial agent was added to wells punched out in agar seeded with the test organism except where indicated.
[a] Using surface inoculation and discs impregnated with the antibacterial agent.

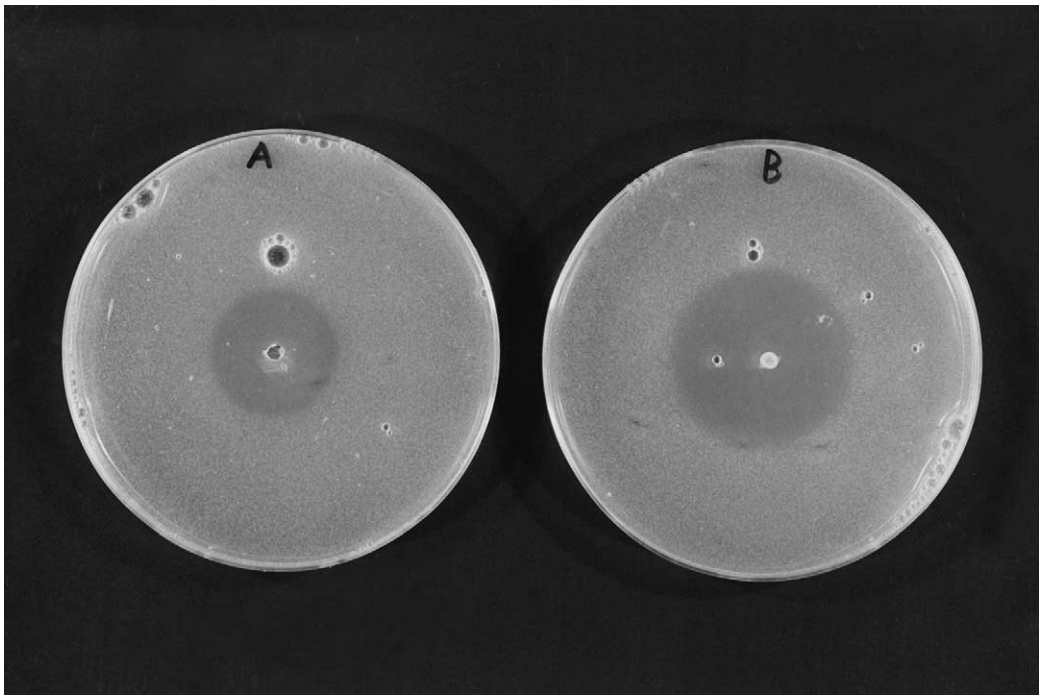

Fig. 4. Inhibition of growth of *S. aureus* by azelaic acid and bis-[(butanoyloxy)methyl] nonanedioate at pH 5.6 in YEGA using the agar inclusion technique. Key: A—azelaic acid (10 mg); B—bis-[(butanoyloxy)methyl] nonanedioate (10 mg).

reduce the antibacterial effect of azelaic acid more than that of its esters.

The activity of (**6**) and azelaic acid demonstrated in vitro, was also seen when topical preparations of either substance (20%, w/w) were applied to the skin. Control plates (diameter = 55 mm) of untreated facial skin or the fingers usually gave too many colonies to count. Application of a thin film of either topical preparation reduced the counts to <10 after 30 min contact time (three analyses). Screening using a contact time of 5–15 min gave a countable number of colonies. The results for the eight runs (facial skin) were as follows: we found that the (**6**) cream (colony forming units, cfu = 106±126) did not give significantly greater reductions in colony counts than Skinoren® (cfu = 32 ± 42); $t(7) = 1.55$, $P > 0.05$. The results for finger dabs (10–15 min contact time), where two fingers on each hand were treated with the ester preparation and two with Skinoren®, also did not show significant differences: cfu = 38 ± 37 (ester), cfu = 27±40 (Skinoren®); $t(6) = 0.91$, $P > 0.05$, A greater number of data points is required to draw conclusions as to the relative efficacies of the two formulations against cutaneous microbes. The results of skin testing with the ester-based cream were somewhat disappointing, given the performance of (**6**) in the in vitro tests described above. However, there are some important aspects of the skin challenge test which need further comment. Firstly, none of the co-workers are acne sufferers, and thus to what extent the test results reflect the situation for the product's target group is not known. In addition, use of an ester-based formulation in the treatment of dermatosis, would require further studies aimed at developing a cream base promoting optimal activity of the ester when applied to the skin. Twenty-two colonies of bacteria which survived contact with the antibacterial cream, were subcultured from contact plates onto YEGA (pH 5.6)

Table 2
MIC values for azelaic acid and its ester bis-[(butanoyloxy)methyl] nonanedioate (**6**) at pH 5.6

Antibacterial (mg/ml)	Bacterial species					
	S. aureus		*S. epidermidis*		*P. acnes*	
	Ester	Aa[a]	Ester	Aa	Ester	Aa
4.5	0	0	0	0	0	0
1.5	0	+	0	++	0	++
0.75	0	++	0	+++	0	+++
0.25	+	+++	+++	+++	+++	+++
0.10	+++	+++	+++	+++	+++	+++
0	+++	+++	+++	+++	+++	+++

[a] Aa: azelaic acid.

containing 0.2–0.7 mg/ml of azelaic acid or (**6**). Of these only one grew on agar containing 0.7 mg/ml of the ester. Whereas 11 clones showed some or good growth on plates containing the equivalent concentration of azelaic acid. This seems to indicate that other factors, such as the composition of the cream base, mitigate the antibacterial activity of the ester.

Another potential anti-acne prodrug of azelaic acid [bis(*o*-carboxyphenyl ethyl ester)nonanedioate] has been evaluated for percutaneous penetration and dermal metabolism with promising results in vivo (Sintov et al., 2002). These findings taken together with the demonstration of good antimicrobial activity (present study), clearly indicate that prodrugs of azelaic acid are promising alternatives in the treatment of acne.

We conclude that (**6**) has interesting antibacterial properties which are worthy of further investigation in clinical trials of a relevant group of test subjects. A wider range of esters of azelaic acid will now be screened for antibacterial activity. We will also investigate the effect of different cream-base compositions on the antibacterial activity of esters using contact plate analysis of the skin.

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