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Mechanisms of action of skin penetration enhancers/retarders: Azone and analogues

Jonathan Hadgraft[a,*], James Peck[b], Dafydd G. Williams[a], W. John Pugh[a], Geoffrey Allan[1,b]

[a] *The Welsh School of Pharmacy, University of Wales College of Cardiff, Cardiff CF1 3XF, UK*
[b] *Whitby Research Inc., 2801 Reserve St., PO Box 27426, Richmond, VA 23261-7426, USA*

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Abstract

The modifying effect of Azone and five analogues on diffusion of metronidazole through isolated stratum corneum (SC) is reported. All enhance diffusion except N-0915, which is a retarder. Enhancement ratios (amount penetrating with modifier/amount from control) at 40 min are Azone 6.7; N-0539 6.4; N-0253 3.4; N-0721 1.4; N-0131 1.1; N-0915 0.2. The sulphur analogue of Azone (N-0721) is much less effective than Azone itself, and the short hydrocarbon chain in N-1031 renders it ineffective. Similar results using diethyl *m*-toluamide as penetrant suggest that this effect of N-0195 is non-specific. Azone expands while N-0915 condenses monolayers of dipalmitoyl phosphatidylcholine (DPPC). The phase transition temperature (T_m) at $\sim 40°C$ of multilamellar DPPC liposomes is lowered by the enhancers in rank order of their enhancing abilities, while N-0915 increases T_m. Thus, modifier activity might be related to fluidising effect on lipid lamellae. A mechanism of modifier action based on alteration of the lateral bonding within SC lipid lamellae is proposed, based on molecular modelling of Azone and N-0915 and their hydrogen bonding capacities to cerebrosides.

Keywords: Stratum corneum; Penetration modifiers; Azone analogues; Hydrogen bonding; Ceramide; Liposome

1. Introduction

There has been considerable interest over the past few years in the development of skin penetration enhancers. Mechanisms of action have been investigated but it has been very difficult to correlate enhancer effects through structure activity relationships. Many of the publications on en-

* Corresponding author.
[1] Current address. Insmed Pharmaceuticals Inc., 800 East Leigh Street, Richmond, VA 23219, USA

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hancer mechanisms suggest that they enter the skin lipids and create disorder. A diffusing penetrant therefore experiences a more fluid environment and permeates more rapidly. The activity of the enhancer may be expected to be a function of its molecular geometry and charge distribution. In view of the developments in molecular graphics it should be possible to examine the effects that the molecular shape and charge distribution have on activity, provided a group of structurally related enhancers are studied (Hoogstraate et al., 1991; Brain and Walters, 1993). It is also theoretically possible that some agents exist which will enter the skin and impart order to the skin lipids thus making the stratum corneum more impermeable. This has obvious implications where it is desirable for agents to reside at or in the skin surface and not penetrate sufficiently to elicit systemic effects. Examples within the cosmetic area (UV filters, etc.) and the agrochemical industry (pesticides) are more obvious cases, however there will be some therapeutic agents where specific targeting to the stratum corneum is desirable.

In this study the effect of some structurally related Azone analogues have been examined from a variety of standpoints. Since Azone® is known to interact with structured lipids, the first investigations have examined the ways in which the enhancer molecules interact with model membranes. Two systems have been chosen for the examination since they have been used in the past: firstly, a study of the effect of the enhancer on the phase transition properties of dipalmitoyl phosphatidylcholine (DPPC) multilamellar vesicles, and secondly, the interaction with a DPPC monolayer using a Langmuir trough. After the studies with a model membrane the effect of the enhancers on in vitro human skin permeability was studied using the model permeant metronidazole. Finally, the enhancer effects were examined in the light of the experimental results and their structures, charge distributions predicted from commercially available molecular graphics computer packages and hydrogen bonding potentials.

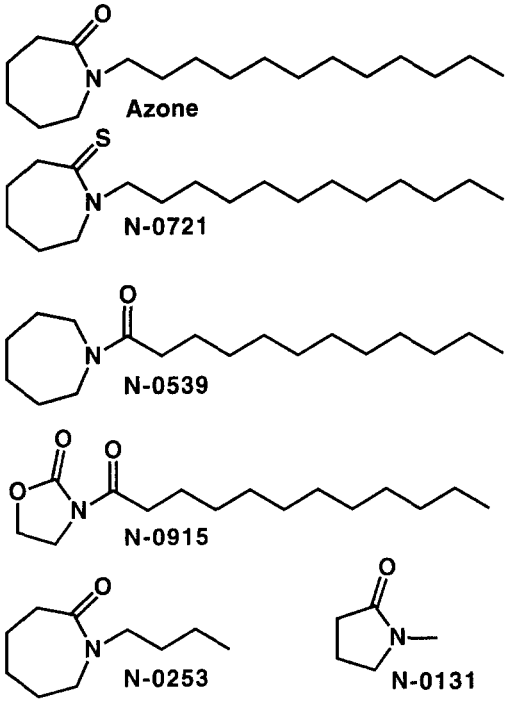

Fig. 1. Azone analogues used in the studies.

2. Materials and methods

Molecules based on the structure of Azone, synthesised and supplied by Whitby Research are shown in Fig. 1. They were used without further purification.

Phase transition temperature determinations were conducted using the technique described by Beastall et al. (1988) DPPC (99% purity) was purchased from Sigma, Poole, UK, and used without further purification. The experiments were conducted five times and the standard deviations were always less than 5% of the mean. The error bars have been omitted from Fig. 2 for clarity.

The surface pressure isotherms were measured on an automated Langmuir film balance (Nima Technology, Coventry, UK) using the techniques described by Lewis and Hadgraft (1990).

Skin diffusion experiments were conducted in all glass Franz type diffusion cells with a surface area of approximately 0.5 cm^2. The receptor

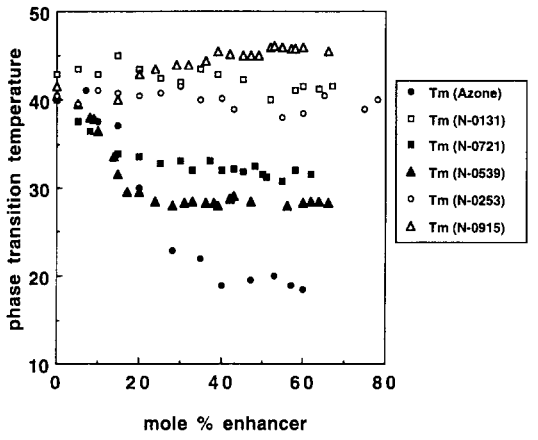

Fig. 2. The effect of the different enhancers on the phase transition temperature of multilamellar DPPC liposomes. The experimental results are the mean of at least five determinations with a standard deviation of less than 5% of the mean value.

phase, pH 7.4 phosphate buffered saline (containing 0.01% phenyl mercuric chloride), was thermostatted at 37°C and the measured skin temperature was 30 ± 2°C. The skin and receptor phase were left in contact for 1 h prior to any treatment. After this initial equilibration time the skin was pre-treated with either 50 μl of ethanol or 50 μl of a 1% ethanolic solution of the enhancer under investigation. After 2 h the donor compartments were treated with 100 μl of an ethanolic solution of metronidazole (5 μmol/ml). At appropriate time intervals, 0.5 ml of the receptor phase were removed and assayed for metronidazole. An equal volume of fresh receptor medium was added to the receptor phase. Permeation experiments were conducted over a 48 h period with four skin samples from the same subject, four controls were run for direct comparison.

The assay procedure used an Apex ODS II column, particle size 5 μm (Jones Chromatography, Hengoed) 25 cm long, i.d. 4.6 mm. The column was eluted isocratically with a mobile phase of 0.05 M sodium acetate: methanol (55:45) at a flow rate of 1 ml/min, monitoring at 320 nm. The retention time under these conditions was 3.85 min.

Computer graphics were produced on an Apple Macintosh Centris 650 using Nemesis software (Nemesis version 2.0, 1992, Oxford Molecular, Oxford, UK). Partial charges were calculated, and the structures minimised following preliminary conformational analysis to avoid local energy minima.

3. Results and discussion

The results for the effect of the enhancers on the phase transition temperature are shown in Fig. 2. On the basis of their ability to reduce the phase transition temperature of DPPC (as judged by the tangents to the curves at low enhancer concentrations) the different enhancers can be ranked: N-0539 ~ Azone > N-0721 > N-0253 = N-0131 > N-0915. It is interesting that one of the compounds (N-0915) increased the phase transition temperature implying that it was inserting into the DPPC bilayers of the liposome and creating order, and hence chain rigidity, rather than disorder. The two compounds with relatively short alkyl chains (N-0253 and N-0131) had no effect on the phase transition temperature. One of these, N-0131 (N-methyl pyrrolidone), has been investigated in the past (Southwell and Barry, 1984) and shown to be a penetration enhancer in skin diffusion experiments. It is suggested that its action is one of solvency rather than one in which the diffusional barrier of the stratum corneum lipids is lowered.

The experiments with the monolayers of the modulators were conducted to establish whether or not there were any specific interactions between the modulators and the DPPC, and to determine the areas per molecule. Stable DPPC-modulator mixed monolayers could only be formed between DPPC and Azone or N-0915. In experiments with the remaining structures the isotherms were either unstable or not sufficiently reproducible to be reported here. This instability may result from solubility problems with the modulator dissolving in the sub-phase. Fig. 3 shows a comparison between Azone and N-0915. The results for Azone alone are similar to those published previously by Lewis and Hadgraft (1990).

The area per molecule of Azone is comparable to that of DPPC whereas N-0915 is smaller. Fig. 3 shows that neither Azone nor N-0915 mixes ideally in the monolayer. In the case of Azone there is an expansion of the area per molecule compared to ideality, whereas for N-0915 there is an indication of compression, i.e. the molecules of N-0915 pull the DPPC molecules together by favourable molecular interactions. The reasons for this will be discussed when the molecular graphics representations of the enhancers are described later. From the data described above it may be anticipated that Azone will insert itself into skin lipids, force their constituent molecules apart and increase the skin permeability. On the other hand, N-0915 may be expected to condense the skin lipids making them less permeable. The other enhancers may be expected to act on the skin in a similar way to the interactions with the structured DPPC molecules, as described by the phase transition temperature experiments.

In order to assess the effects on skin, experiments were conducted on full thickness human skin. Because of the inherent variability of skin, the experiments were conducted such that each enhancer was compared to a control on skin from the same donor. The variability of skin can be seen by comparing the permeability of the model permeant, metronidazole in the control cells. The data are compared in Fig. 4.

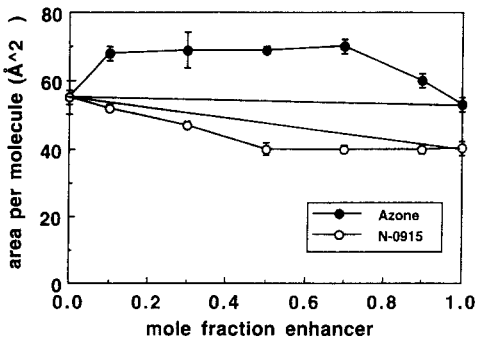

Fig. 3. Average area per molecule as a function of the mole fraction of the enhancer incorporated into a DPPC monolayer. The surface pressure was 15 mN/m. The tie lines connecting the values at 0 and 1.0 mole fraction represent the expected areas per molecule assuming no interaction.

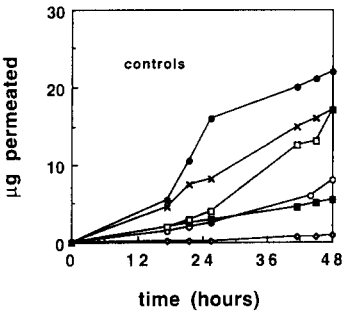

Fig. 4. A comparison of the amount of metronidazole permeated across in vitro human full thickness skin from control solutions.

This variability is not unreasonable in magnitude (Southwell et al., 1984) but highlights the difficulty of conducting comparative studies of skin permeation.

The results for the different modulators are shown in Fig. 5. Despite the variability in the controls, it is possible to see the relative effects that treatment with the various structures has on the permeability of the skin. Considering Azone as the standard, it is seen that N-0539 has a higher enhancing activity for this permeant. This should be considered in conjunction with the data presented in Fig. 2. Although at high concentrations Azone was more effective at lowering the phase transition temperature of the DPPC liposomes, at low enhancer concentration N-0539 was more effective.

N-0131 and N-0253 had no effect on the phase transition temperature and, up to 24 h, had no effect on the enhancement of metronidazole permeation. N-0253 did appear to act as an enhancer after 24 h. The reasons for this are unclear, but could be a result of breakdown of the integrity of the skin over longer times. N-0721 had some effect on lowering the phase transition temperature (< Azone) of the DPPC liposomes but had no effect on skin permeability. In general, there was a rank order correlation between the phase transition temperature effects and the increased permeability. More interestingly, the compound N-0915 which increased the phase transition temperature of the liposomes decreased the permeability of the skin to metronidazole.

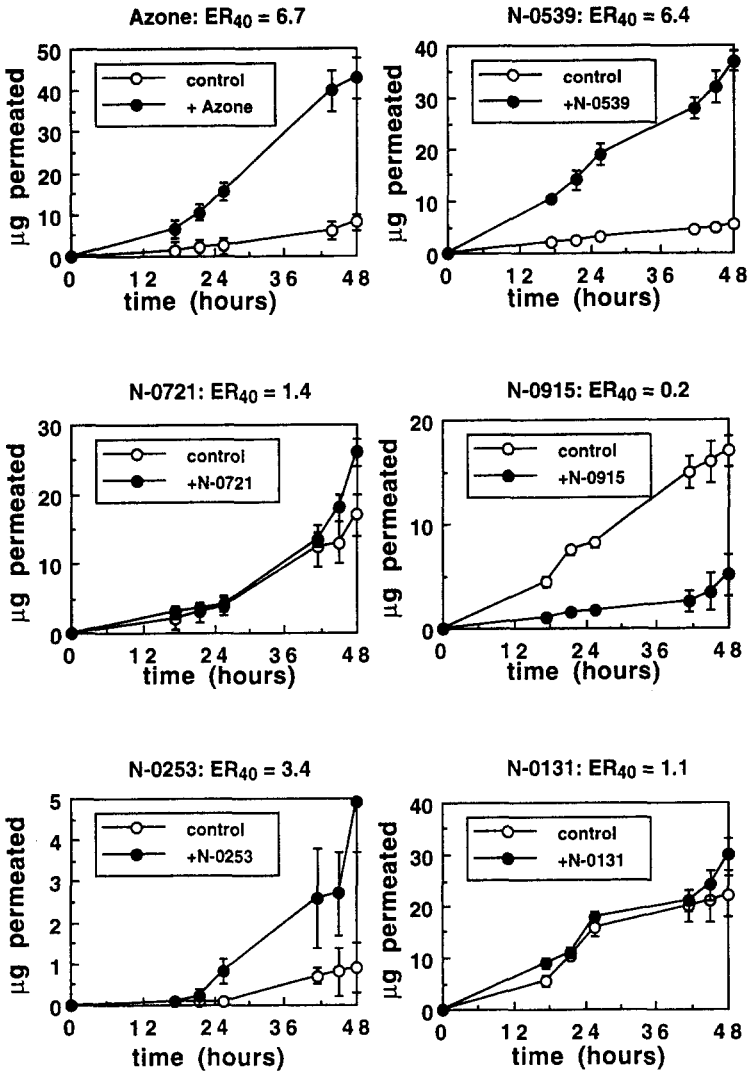

Fig. 5. A comparison of the effects of the different enhancers on the permeation of metronidazole through full thickness human skin. Enhancement ratios (ER) at 40 h are shown.

It is possible that this effect is specific to metronidazole, and, in order to examine whether or not N-0915 could retard the penetration of a material with potentially toxic effects, the penetration of the mosquito repellent diethyl *m*-toluamide (DEET) was examined. It is interesting to note that this material has also been examined as a potential penetration enhancer. Similar experiments were conducted on full thickness human skin with DEET being used in place of metronidazole. At 2 h after pre treatment, 100 μl dose of a test solution of 1.5 mg/ml of DEET in ethanol was applied to the skin surface and allowed to evaporate. This dose of 0.3 mg/cm^2 exposed skin has previously been shown to determine the effective duration of mosquito repellents on human skin (Reifenrath and Robinson, 1982). The results are shown in Fig. 6 and indicate that DEET and metronidazole absorption are both retarded to a very similar degree.

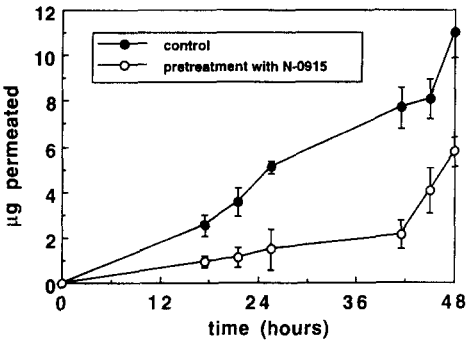

Fig. 6. Retardation of absorption of the mosquito repellant, DEET through full thickness human skin, $n = 4$, mean $\pm$ S.D.

It has been hypothesised that the enhancement effect of Azone arises from its ability to exist in a bent 'soup spoon' conformation (Lewis and Hadgraft, 1990; Hoogstraate et al., 1991) where the ring was at an angle to the lipophilic chain (Fig. 7). It was argued that the increased energy of the angled conformation - which pertains to a gas phase molecule - would be compensated by removal of the hydrophobic ring methylene groups from the polar region of the lamella back towards the lipophilic hydrocarbon chain region. The minimum energy conformation (5.2 kcal) is shown in Fig. 7 and it can be seen that it is essentially planar and would be expected to intercalate between SC ceramides with a minimum of spatial disruption. The 'bent spoon' conformation 2 is obtained by setting the C23-C22-C16-N1 bond to 62° and has an energy of 6.1 kcal. (Nemesis does not permit setting the torsion angle O-N1-C16-C22 (1°) to the 10° specified by Hoogstraate et al. (1991). The energy difference ($\Delta E \approx 1$ kcal) corresponds to a high probability level of about 0.2 for the existence of the 'bent spoon' at 37°C. However, it must be borne in mind that intercalation into a liquid bilayer structure of packed ceramide molecules will provide additional resistance to the existence of this higher energy formation. In view of this we offer an alternative hypothesis for the enhancing actions of Azone analogues based on competition for H-bonding sites between SC lipids. This hypothesis would also explain the retarding effect of N-0915.

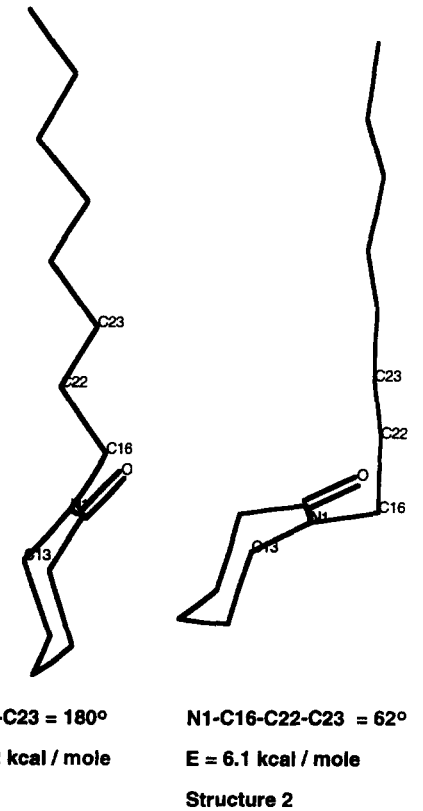

Fig. 7. Conformations of Azone. (Only eight chain hydrocarbons shown). C13-N1-C16-C22 = 90° for both forms.

The nature of the forces holding the modifiers within the SC (or liposome) bilayers is not known with certainty, but H-bonding between head groups (Pascher, 1976) is accepted as being an important factor in stabilising ceramide bilayers. Pascher considered the intermolecular bonding to be between -OH groups. Pascher and Sundell (1977) attributed changes in thermal transitions on incorporation of cholesterol to be due, at least in part, to reorientation of these hydrogen bonds. Jackson et al. (1988) noted very specific interaction between polar head groups of galactocerebrosides and cholesterol, and low concentrations of cholesterol were sufficient to disrupt the cerebroside–cerebroside interaction (Johnston and Chapman, 1988; Wiedmann and Salmon, 1991). These conclusions have been reinforced by the findings of Mizushima et al. (1995) working with lamellae formed from synthetic ceramides.

Recent evidence (Abraham et al., 1995; Potts and Guy, 1995; Roberts et al., 1996) suggests that the H-bonding power of the penetrant is a major determinant of penetration. It is reasonable, therefore, to infer that modification of H-bonding within the natural SC lipids is a possible mode of action of modifiers. The composition of the SC lipids given by Wertz (1992) suggests that the most powerful H-bonding lipid is ceramide 6, which has four secondary alcohol and one secondary amide groups. Thus binding between ceramide 6 molecules should represent the strongest intermolecular binding possible between SC lipids. We therefore set out to estimate the effect of modifier on binding between two ceramide 6 molecules, on the basis that this would represent the minimal effect that the modifier could exert.

Because of the constraints imposed by being packed into lamellae, the ceramides are bent so that their polar groups line the interlamellar region (Suzuki et al., 1985), and intralamellar bonding is possible between adjacent alcohol groups (Pascher and Sundell, 1977).

Abraham (1993) gives the H-bonding acceptor (α) and donor (β) strengths for secondary alcohols as 0.33 and 0.56, respectively. The strength of the intramolecular bond formed can be estimated as $(0.33 \times 0.56) = 0.18$ (arbitrary units).

Explanation of the mechanism of penetration modification on SC in terms of the observed physical effects of the compounds on DPPC liposomes is bound to be speculative since DPPC does not occur in the SC. The importance of apparently minor variations in chemical substituents in materials incorporated into bilayers was demonstrated by Brasseur et al. (1983) who incorporated miconazole, ketoconazole and deacylated ketoconazole into DPPC vesicles. They found that the small change in the drug molecule involved in deacylating ketoconazole completely altered its effect on the bilayer. Ketoconazole, occupying a cross sectional area of 30 Å^2, had little effect on the transition temperature. The orientation within the bilayer of the deacylated drug was reversed with the piperazine moiety turned toward the aqueous phase. It occupied 90 Å^2 and had a dramatic destabilising effect on the bilayer, seen as a reduction in transition temperature. Similar effects on the packing of DPPC molecules are seen.

The position of a modifier in the lamellar structure will depend on the balance between its hydrophilic and lipophilic natures. Bouwstra et al. (1992) showed that Azone analogues with six to twelve carbons in the hydrophobic chain were intercalated in the bilayer, but surprisingly the hexyl analogue caused no disordering of the lipid structure. Compounds with small hydrocarbon chains (N-0253 and N-0131) might be expected to be situated well into the polar region.

The long hydrocarbon chain of the other modifiers thus results in the possibility of the

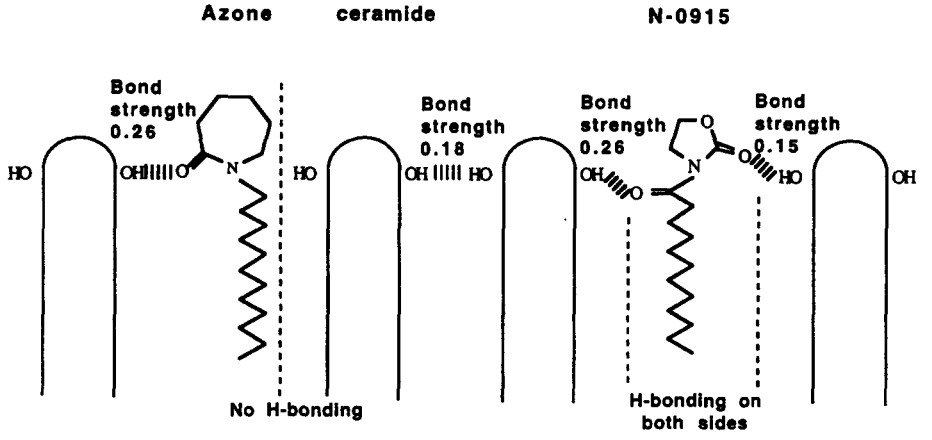

Fig. 8. Proposed hydrogen bonds between ceramides and modifier molecules.

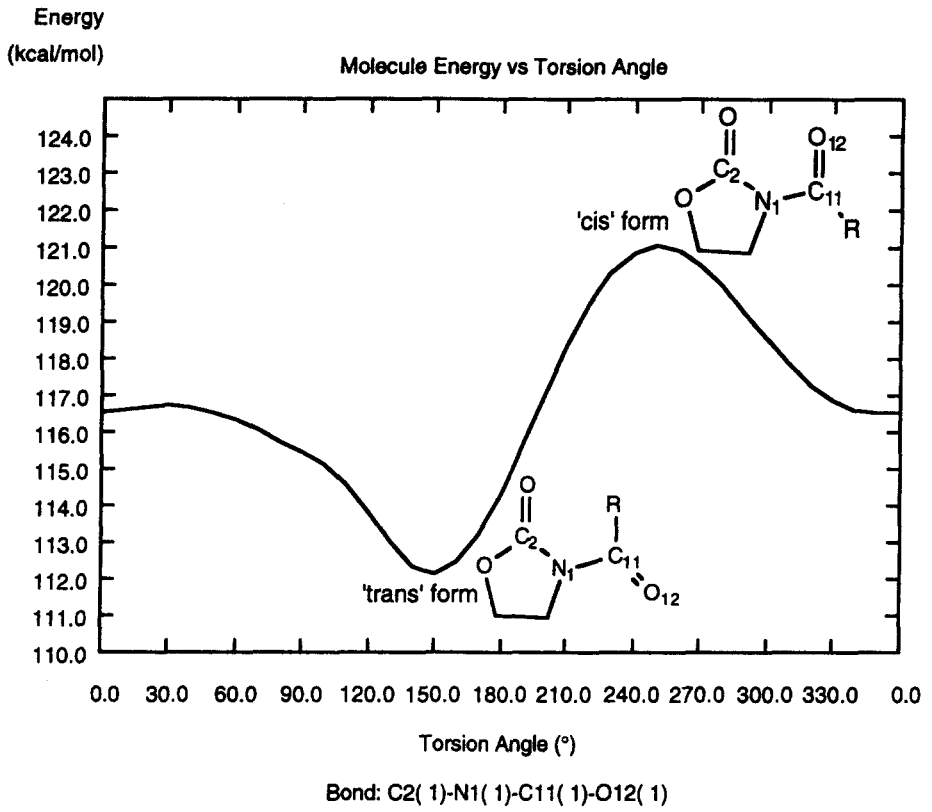

Fig. 9. Energy profile for N-0915.

modifier intercalating between adjacent ceramide molecules. To do this it must additionally compete effectively for H-binding sites in the head groups of the ceramide molecule. From Abraham's data both Azone and N-0539 should have $\alpha = 0$ and $\beta \approx 0.78$ units giving a H-bond strength to ceramide alcohol of 0.26 units (i.e. 0.78×0.33). The strength of ceramide–ceramide bonds, i.e. between two secondary alcohols ($\alpha = 0.33$, $\beta = 0.56$) would be 0.18 units. It is therefore possible that Azone and N-0539 can bind firmly to a ceramide molecule, the displaced ceramide now being unbound (Fig. 8). Thus, a region of fluidity appears in the lamella enabling enhanced penetration. The sulphur analogue of Azone, N-0721, would have a binding strength ≈ 0.15 units and so would be less efficient at displacing ceramide.

The retardant, N-0915, has extra oxygen atoms. The conformation where all oxygen atoms are on the same side of the ring is energetically unfavourable (Fig. 9). There is 9 kcal more energy in this *cis* form, rather than the *trans* form where the chain oxygen is the other side of the ring, giving a probability for its existence of only 5×10^{-7} at 37°C. This favoured conformation thus has H-bonding groups potentially available to adjacent ceramide head groups on either side, raising the possibility of cross linking to both. Condensation of the lamella would be expected if the N-0915–ceramide bonds were stronger than ceramide–ceramide bonds (0.18 units). For the head group structure of N-0915, α and β values are not reported. As an approximation we have calculated them as ester and tertiary amide groups for which Abraham (1993) gives $\alpha = 0$, $\beta = 0.45$; $\alpha = 0$, $\beta = 0.78$ respectively, resulting in H-bonding of 0.15 and 0.26 units to secondary alcohols on adjacent ceramides (Fig. 8). It therefore seems possible that

N-0915 can act as a retarder by producing condensation via more powerful H-bonding within the plane of the polar head groups.

If this hypothesis is correct it should enable the rational design of more efficient enhancers and retardants.

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