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Marine lipids as building blocks for soft quaternary ammonium compounds and their antibacterial activity

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Environmental friendly antibacterial agents have to degrade relatively rapid to non-toxic and inactive products after they have had their desired effect. Environmental friendly quaternary ammonium agents were designed according to Bodor's soft drug approach and evaluated *in vitro*. Structure-activity relationship (SAR) studies showed that the antibacterial activity of a given soft agent will only be acceptable if its chemical stability is adequate to allow the agent to express its activity for sufficient duration of time. However, the studies also showed that increasing the lipophilicity of a chemically labile antibacterial agent could increase its potency. Two of the lipophilic quaternary ammonium antibacterial agents evaluated had minimum inhibitory concentration (MIC) against *Staphylococcus aureus* as low as 2 μg/ml and estimated degradation half-life less than 4 to 6 days at room temperature. Decreased MIC could only be obtained by increasing the degradation half-life of the agents.

1. Introduction

Various marine products, such as fish oils, are rich in unsaturated fatty acids. Approximately 98% of the refined cod-liver oil consists of triglycerides, the rest is unsaponifiable matter, free fatty acids, monoglycerides and diglycerides. The acid part of the glycerides consists mainly of various unsaturated fatty acids, including the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Previously, we have extracted the fatty acids from the cod-liver oil and shown that this extract enhances transdermal and buccal drug delivery (Loftsson et al. 1995; 1997a; 1998; Tsutsumi et al. 1998a; 1998b; Másson et al. 2000a). The fatty acid profile of the extract is almost identical to that of the oil (Table 1). About 17% of the extract consists of saturated fatty acids, mainly palmitic acid (10.4%), and the rest is unsaturated fatty acids such as oleic acid (16.2%), DHA (11.9%), gondoic acid (9.4%), EPA (9.3%), cetoleic acid (7.8%), palmitoleic acid (6.4%) and *cis*-vaccenic acid (4.4%). The penetration enhancing effect was found to be associated with the unsaturated fatty acid portion of the extract. Interestingly, cod-liver oil itself does not enhance transdermal drug delivery. It is well known that many fatty acids and their monoglycerides possess both antimicrobial and antiviral activity (Kabara et al. 1977; Thormar et al. 1987; Thorgeirsdottir et al. 2003). In an unpublished study we have shown that the fatty acid extract from cod-liver oil (Table 1) has significant antibacterial activity while cod-liver oil itself, containing the same fatty acids in the form of triglycerides, does not affect bacterial growth (Fig. 1, Loftsson et al. 1997b). Furthermore, we have shown that both the fatty acid extract from cod-liver oil and an ointment containing the extract have a notable antiviral effect against herpes simplex virus type 1 (HSV-1) (Loftsson et al. 1998). One per cent fatty acid extract caused a 50,000 fold or greater ($\geq 4.7 \log_{10}$) reduction of viral infectivity in 10 min. Ointment containing 30% extract caused 1.5 million fold ($\geq 6.2 \log_{10}$) reduction of viral infectivity. These results are in agreement with earlier data which showed that long chain unsaturated fatty acids are potent inactivators of HSV-1 and other enveloped viruses (Thormar et al. 1987).

Table 1: Fatty acid composition of triglycerides found in cod-liver oil and its fatty acid extract (Loftsson et al. 1995)

Fatty acid		Composition (%)	
Name	Number	Cod-liver oil	The extract
Myristic acid	14 : 0	3.6	3.6
Palmitic acid	16 : 0	10.5	10.4
Palmitoleic acid	16 : 1 n-7	6.5	6.4
Stearic acid	18 : 0	2.6	2.6
cis-Vaccenic acid	18 : 1 n-7	4.4	4.4
Oleic acid	18 : 1 n-9*	16.3	16.2
Linoleic acid	18 : 2 n-6	1.6	1.5
Moroctique acid	18 : 4 n-3	2.4	2.4
cis-11-Eicosenoic acid	20 : 1 n-7	0.4	0.5
Gondoic acid	20 : 1 n-9	9.6	9.4
Gadoleic acid	20 : 1 n-11	1.5	1.6
Eicosapentaenoic acid (EPA)	20 : 5 n-3	9.6	9.3
Erucic acid	22 : 1 n-9	0.6	0.6
Cetoleic acid	22 : 1 n-11	7.7	7.8
Clupandonic acid	22 : 5 n-3	1.4	1.4
Docosahexaenoic acid (DHA)	22 : 6 n-3	12.5	11.9

* And linolenic acid (18 : 3 n-3) that was not separated from 18 : 1 n-9 in the GC system. Cod-liver oil usually contains less than 1% linolenic acid.

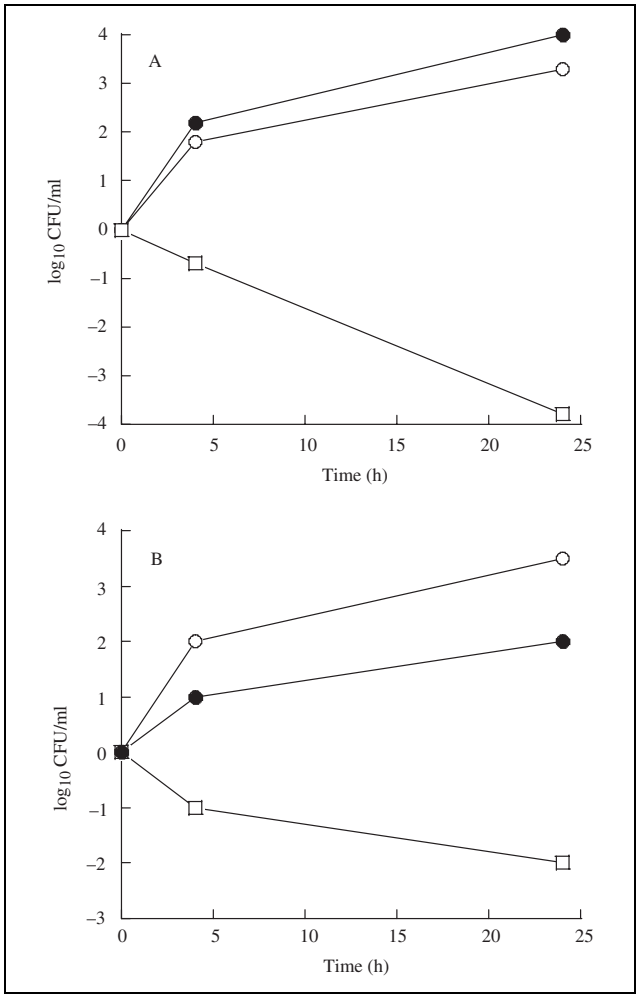


Fig. 1: The antibacterial effect of cod-liver oil (○) and fatty acid extract from cod-liver oil (□) against *Staphylococcus aureus* (A) and *Streptococcus pyrogenesis* (B). The concentration of both the cod-liver oil and the extract was 3.25 mg/ml. The solvent was ethanol (●)

Previously, we have used some of the fatty acids extracted from cod-liver oil as pro-moieties in prodrug design for dermal and transdermal delivery of drugs (Thorsteinsson et al. 1999; Másson et al. 2000a; 2000b; Thorsteinsson et al. 2000; 2002). Here we show how these same fatty acids can be used as building bocks in the synthesis of soft antibacterial agents.

2. Investigations, results and discussion

2. Soft antibacterial agents

It is believed that bacteria acquire resistance in two stages. In the first stage the bacteria mutates to obtain resistance to low levels of the antibacterial agent and in the second stage the bacteria achieves higher levels of resistance through further genetic mutation and gene transfer from other bacteria species. Thus, sustained low concentrations of an antibacterial agent will increase bacterial resistance. Overuse of antibiotics for treatment of human infections has contributed to the increase in antibiotic resistance (Thorsteinsson et al. 2003a). However, as much as 50% of the total antibiotic production is used for purposes other than human therapy. Antibacterial agents fed to animals and fish, and sprayed on fruit trees, give rise to resistant bacteria. Even disinfectants commonly used in household products, e.g. triclosan, can contribute to increase in resistant bacteria (Levy 1998; McMurry et al. 1998).

Hard drugs have been defined as drugs that are biological active and non-metabolizable or drugs that are metabolized to form biologically active compounds (Ariëns 1980). Quaternary ammonium compounds, such as benzalkonium chloride and cetylpyridinium chloride, are hard antibacterial agents. Their toxicity limits their use in humans and animals, and their chemical stability limits their use for general environmental sanitation. Furthermore, due to their chemical stability they may induce selective antimicrobial pressure and bacterial resistance (Thorsteinsson et al. 2003a). About 25 years ago Bodor developed the soft drug approach to drug design (Bodor et al. 1980; 1984). He defined soft drugs as drugs that are characterized by predictable and controllable *in vivo* destruction (i.e. metabolism) to form non-toxic products after they have achieved their therapeutic role (Bodor et al. 1980; 1997). Safer drugs are obtained with an increased therapeutic index by integrating metabolism considerations into the drug design process. Likewise, environmental friendly compounds can be obtained by integrating chemical degradation considerations into design of, for example, antibacterial agents. One of the first applications of this approach were the soft analogues of cetylpyridinum chloride (Bodor et al. 1980). The soft analogues possess similar physicochemical properties as cetylpyridinium chloride, including antibacterial properties, but they were about 40-times less toxic when given orally to mice (Fig. 2). However, the minimum inhibitory concentration (MIC) of cetylpyridinium chloride was somewhat lower than that of the soft analogues. Since then several research groups have synthesized and tested soft antibacterial agents (Thorsteinsson et al. 2003a). These agents include soft betaine esters (Ahlström et al. 1999b; 1999a), bis-quaternary ammonium compounds (Agharkar et al. 1976; Devinsky et al. 1991; 1996) and carnetine esters (Calvani et al. 1998). We have synthesized 35 different soft quaternary ammonium compounds and tested their chemical stability, lipophilicity and antibacterial activity (Thorsteinsson et al. 2003b). The selection of building blocks was partly based on compounds found in marine lipids, with polar quaternary ammonium head groups and chemically labile spacers (see Fig. 3). The structures of some of the compounds synthesized, as well as their physicochemical properties and antibacterial activity, are shown in Table 2. There does not appear to be much correlation between the anti-

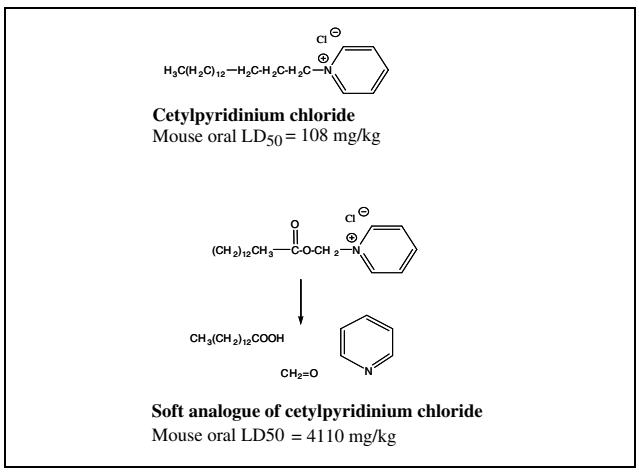


Fig. 2: Structures and toxicities of cetylpyridinium chloride and its soft analogue

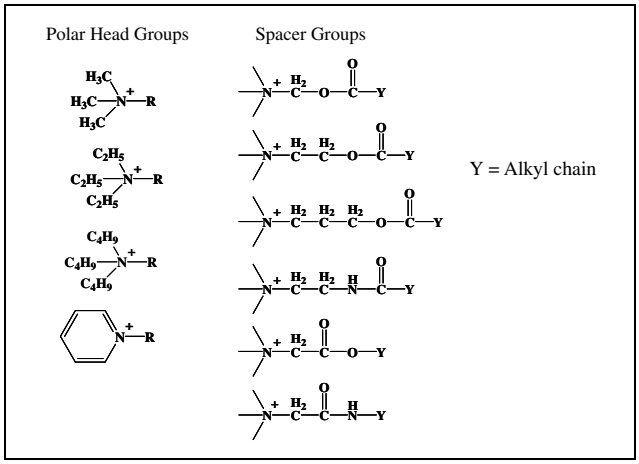


Fig. 3: The soft quaternary ammonium antibacterial agents were formed by linking three building blocks together via covalent bond, i.e. a quaternary ammonium head group, a chemically labile spacer group and a lipophilic alkyl chain

microbial activity and the lipophilicity of the compounds, or their antibacterial activity and chemical stability (Fig. 4). However, when all those factors were combined in one structure activity relationship (SAR), there appears to be a strong relationship between all these three parameters. The relationship follows the equation:

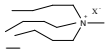
$$\text{Log}\,(1/\text{MIC}) = 1.316\,\text{Log}\,t_{1/2} + 0.350\,\text{C Log P} - 3.202$$
$$(\text{stdev.} = 0.91),$$
$$R^2 = 0.73,\ p = 0.004$$

where $t_{1/2}$ is the half-life in hours for the hydrolysis of the compounds in aqueous buffer solution at pH 6.0 and 60 °C, and Clog P is the logarithm of their calculated or determined octanol/water partition coefficient. Comparison of measured values and calculated values is shown in Table 2. The correlation shows that the antibacterial activity of a given soft agent will only be acceptable if its chemical stability is adequate to allow the compound to express its activity for sufficient duration of time. However, the study also shows that increasing the lipophilicity of a chemically labile antibacterial agent can increase its potency. The quaternary ammonium antibacterial agents were shown to be much more potent than the corresponding free fatty acids.

2.2. Characteristics of a soft quaternary antibacterial agent

The hydrolytic rate constants of the antibacterial agents were determined and the enthalpy and entropy of activation was estimated to be on the average about 75 KJ/mol and −186 J/mol/K, respectively. Based on these values it can be estimated that a compound having $t_{1/2}$ of 3 h at 60 °C will have $t_{1/2}$ of 1 day at 37 °C, 10 days at 20 °C and 30 days at 10 °C. If the inactivation time is defined as ten $t_{1/2}$ (i.e. when 99.9% of the antibacterial agent has degraded) then the inactivation time will be approximately 10 days, 100 days and one year at 37 °C, 20 °C and 10 °C, respectively. In other words, if soft antibacterial agents are defined as those inactivated in less than 10 days at 37 °C or 100 days at room temperature then they will have to have $t_{1/2} \leq 3$ h at pH 6.0 and 60 °C. Further-

Table 2: Soft quaternary antibacterial agents, their minimum inhibitory concentration (MIC) against *Staphylococcus aureus*, the logarithm of determined or calculated partition coefficient (ClogP), the logarithm of their half-life ($t_{1/2}$) in hours in aqueous buffer solutions at pH 6.0 at 60 °C, and their observed and predicted antibacterial activity (Thorsteinsson et al. 2003b)

Head group	Spacer	Alkyl chain	MIC (μg/ml)	C log P	log $t_{1/2}$	log (1/MIC)	
						Observed	Predicted
	$-CH^2CH^2-$	$-OOC(CH^2)^{10}CH^3$	16	3.41	0.53	−1.20	−1.31
	$-CH_2CH_2-$	$-OOC(CH_2)_{10}CH_3$	16	4.90	0.52	−1.20	−0.80
	$-CH_2CH_2-$	$-OOC(CH_2)_{14}CH_3$	2	6.86	0.59	−0.30	−0.02
	$-CH_2-$	$-OOC(CH_2)_{10}CH_3$	50	2.03	0.31	−1.51	−2.90
	$-CH_2CH_2-$	$-OOC(CH_2)_{10}CH_3$	6	1.97	0.33	−0.90	−2.08
	$-CH_2CH_2-$	$-OOC(CH_2)_6CH_3$	500	0.01	0.62	−2.69	−2.38
	$-CH_2CH_2-$	$-OOC(CH_2)_{14}CH_3$	1	3.94	1.10	0.00	−0.37
	$-CH_2CH_2-$	$-OOC(CH_2)_{16}CH_3$	1	4.92	0.90	0.00	−0.29
	$-CH_2CH_2-$	$-OOC(CH_2)_7CH{=}CH(CH_2)_7CH_3$	8	4.70	1.17	−0.90	−0.02
	$-CH_2CH_2-$	$-HNOC(CH_2)_{14}CH_3$	2	3.20	Stable	–	–
	$-CH_2CH_2CH_2-$	$-OOC(CH_2)_{10}CH_3$	4	2.46	1.81	−0.60	−0.04
	$-CH_2CH_2CH_2-$	$-OOC(CH_2)_{14}CH_3$	<0.25	4.43	2.36	0.60	1.46
	$-CH_2CO-$	$-OCH_2(CH_2)_4CH_3$	>12,800	−1.13	0.15	−4.10	−3.40
	$-CH_2CO-$	$-OCH_2(CH_2)_{14}CH_3$	12,800	3.78	−0.90	−4.10	−3.06
	$-CH_2CO-$	$-OCH_2(CH_2)_{16}CH_3$	>12,800	4.76	−1.10	−4.10	−2.98
	$-CH_2CO-$	$-HN(CH_2)_{11}CH_3$	4	3.27	0.92	−0.60	−0.85
	$-CH_2CO-$	$-HN(CH_2)_{17}CH_3$	<0.25	4.86	0.81	0.60	−0.43
	$-CH_2CH_2CO-$	$-OCH_2(CH_2)_{14}CH_3$	250	4.27	0.62	0.42	−0.89
	$-CH_2CH_2-$	$-OOC(CH_2)_{10}CH_3$	2	6.37	0.27	−0.30	−0.61
	$-CH_2CH_2-$	$-OOC(CH_2)_{14}CH_3$	2	8.34	0.12	−0.30	−0.13
	Benzalkonium chloride		1	–	Stable	–	–
	Cetylpyridinium chloride		<0.5	–	Stable	–	–

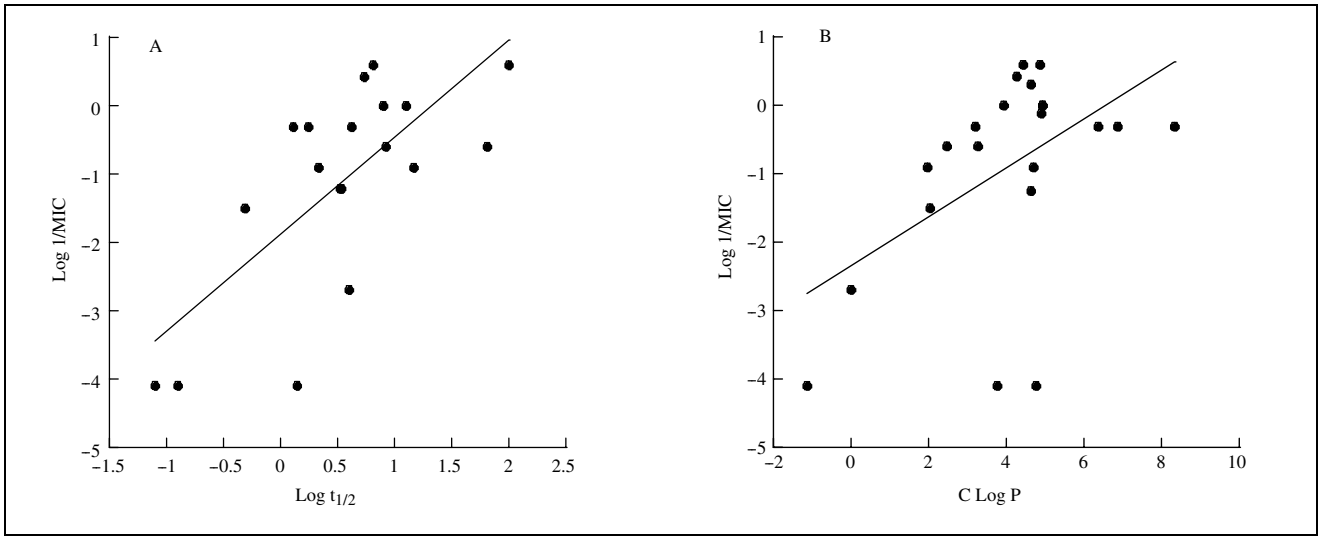


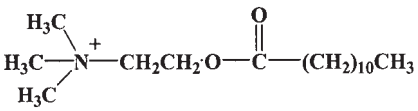
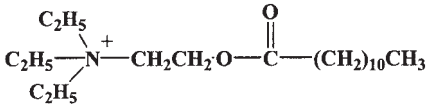
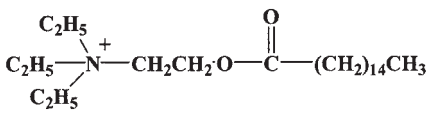
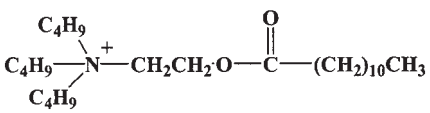
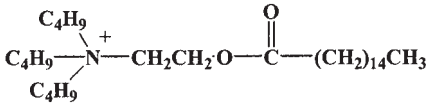
Fig. 4: Structure-activity relationships (SAR), Log (1/MIC) versus Log ($t_{1/2}$) (A) and Log (1/MIC) versus calculated Log P (B)

more, if we define active compounds as those having MIC $\leq$ 10 μg/ml then it can be calculated from the SAR equation that if an agent has $t_{1/2} \leq 3$ h at pH 6.0 and 60 °C ($\log t_{1/2} \leq 0.5$) then its C log P value has to be ≥ 4.5. Based on these definitions only two of the compounds synthesized and evaluated can be regarded as soft quaternary antibacterial agents, with antimicrobial activity comparable to those of benzalkonium chloride and cetylpyridinium chloride (Table 3). None of the pyridinium derivatives tested fulfilled these requirements. Furthermore, MIC less than 2 μg/ml can most probably only be obtained by increasing the degradation half-life of the agents.

2.3. Conclusions

Environmental friendly antibacterial agents have to degrade in the environment to form biologically inactive degradation products, preferably products that can already be found in the environment. In addition their degradation, i.e. deactivation, has to occur within a relatively short time after they have had their desired effect. In other words, the agents have to degrade relatively rapid in the environment but at the same time they have to be potent. However, the potency of soft antibacterial agents will be decreased if they are degraded too rapidly, that is before they can kill bacteria within a given culture. Thus, the

Table 3: Soft quaternary antibacterial agents

Antibacterial agent	$t_{1/2}$ (h)	MIC (μg/ml)*	C Log P
	3.4	16	3.4
	3.3	16	4.9
	3.9	2	6.9
	1.9	2	6.4
	1.3	2	8.3

* Minimum inhibitory concentration (MIC) against Staphylococcus aureus

requirements for rapid deactivation and high potency are contradicting each other. The SAR studies show that potent soft quaternary antibacterial agents can be obtained by increasing the lipophilicity of the agents.

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