ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1975, p. 742-750

Vol. 8, No. 6
Printed in U.S.A.

Antimicrobial Effect of Simple Lipids with Different Branches at the Methyl End Group

KÅRE LARSSON,* BÖRJE NORÉN, AND GÖRAN ODHAM

Department of Food Technology, Chemistry Center, S-220 07 Lund 7, and Ecology Center, University of Lund, S-223 62 Lund, Sweden*

Received for publication 21 May 1975

Various fatty acids of branched nature possess fungistatic and bacteriostatic properties. Some of these, particularly those of *iso*-configuration, strongly enhance the effect of conventional antimicrobial agents that act inside the cell membrane. A relation between this biological effect and the collapse properties of the corresponding monomolecular surface film on water has been observed. In this work, a series of fatty acids with a slightly smaller end group than *iso*-propyl, the ω-cyclopropane fatty acids, as well as one possessing a somewhat larger end group, the *neo*-branched fatty acids, have been examined. The ω-cyclopropane fatty acids were found to be more fungistatic than the *iso*-acids studied earlier. Furthermore, both cyclopropane and *neo*-fatty acids of short chain lengths exhibited synergistic effects in combination with tetramethylthiuramdisulfide.

The antimicrobial activity of various simple lipids and a model study of their mode of action have earlier been reported (8). The strongest biological effects were observed with branched fatty acids. The swelling of hyphae of *Fusarium roseum* by *iso*-fatty acids was interpretated as evidence for an increase in the permeability of the plasma membranes. An expected consequence of this would be the possibility of obtaining a synergistic effect in the presence of other antimicrobial substances which act inside the plasma membrane, and such enhancement effects were also observed.

This paper concerns the antimicrobial activity of other fatty acids, selected with the guidance of our earlier results. The previous work showed that the strongest effect was obtained when the branches were located near the end of the hydrocarbon chains. It was also evident that the size of such an end group should not differ much from an *iso*-methyl configuration. A series of compounds with a slightly smaller end group, ω-cyclopropane fatty acids, as well as one possessing a somewhat larger end group, *neo*-branched fatty acids, therefore was examined.

MATERIALS AND METHODS

Preparation of lipids. Since the fatty acids (except 9-cyclopropanenonanoic acid) have not been previously reported in the literature, their preparation is given in some detail. Detailed physical and chemical characteristics will be published elsewhere.

Neo-fatty acids. A series of fatty acids, the *neo*-isomers of the acids with 16, 18, 20, 22, and 24 carbon atoms, has been synthesized by Sobotka and Stynler (15). The mono-ethyl ester aldehydes of the appropriate dibasic acids were condensed with neohexyl magnesium chloride. Using a different route, involving monoalkylation of methyl pivaloylacetate with ω-iodoesters, Arosenius et al. (1) prepared two members of the series with odd numbers of carbon atoms. Some physical data of 14,14-dimethylpentadecanoic and 20,20-dimethylheneicosanoic acid are given. Since ethyl pivaloylacetate is commercially available, alkylation of this β-keto ester was chosen for the chain-lengthening.

12,12-Dimethyltridecanoic acid. The alkylating agent, methyl 9-iodononanoate, was prepared from methylhydrogen decan-1,10-dioate via the method devised by Barton and Serebryakov (3) and Odham (12). The half-ester (35.6 g [0.165 mol]) yielded 42.2 g of crude methyl 9-iodononanoate. Ethyl pivaloylacetate (21.4 g [0.124 mol]) (Fluka AG, Buchs, Switzerland) was alkylated by refluxing it with 42.2 g of the iodoester in the presence of 76 g of potassium carbonate in 100 ml of dry methyl isobutylketon for 18 h. The reaction mixture was acidified with diluted hydrochloric acid and extracted with ether. After washing, drying, and evaporation, 38 g of crude alkylated keto ester was obtained. Hydrolysis and ketonic cleavage of the alkylation product was effected by adding a solution of 35 g of potassium hydroxide in 45 ml of water and 100 ml of ethanol and refluxing the mixture overnight. The reaction mixture was acidified and extracted with ether. The residue obtained after evaporation of the solvent (25 g) was triturated with 800 ml of light petroleum (boiling point, 40 to 60 C) and 15.3 g of crude keto acid was dissolved.

The keto acid (15.0 g), 15.0 g of potassium hydroxide, 15 ml of 85% hydrazine hydrate, and 125 ml of diethylene glycol were refluxed for 2 h. The con-

denser was removed, and the temperature in the flask was maintained at 230 C for 4 h. After cooling the reaction mixture was acidified and extracted with ether. The extract was washed with water, dried, and evaporated to dryness.

The crude 12,12-dimethyltridecanoic acid was triturated with 200 ml of light petroleum (boiling point, 40 to 60 C) and the soluble material was distilled. The fraction boiling at 150 C (1.3 mm) proved 98% pure by analytical gas-liquid chromatography (GLC) (yield, 5.3 g; melting point, 30.5 to 31.0 C [remelted]).

18,18-Dimethylnonadecanoic acid. The acid was prepared via a mixed anodic coupling (Kolbe electrosynthesis) of 12,12-dimethyltridecanoic acid and methyl hydrogen octan-1,8-dioate (see reference 8). One gram (4.14 mmol)of the *neo*-acid and 0.84 g (4.14 mmol) of the half-ester were electrolyzed. The crude methyl ester obtained (953 mg) was chromatographed on 25 g of silicic acid (Mallinckrodt, 100 mesh) with ether-light petroleum (boiling point, 40 to 60 C) (1:50 vol/vol). After a forerun of hydrocarbon character (148 mg) 300 mg of methyl 18,18-dimethylnonadecanoate was obtained as a viscous liquid. Purity was 97% as indicated by GLC. The free acid had a melting point of 56.0 to 56.6 C (remelted).

ω-Cyclopropane fatty acids. Cyclopropane fatty acids occur frequently in nature, particularly in bacteria (13), protozoa (10), and plants (4). The cyclopropane ring is usually remote from the hydrocarbon end group of the fatty acid. The preparation of a series of such compounds is described by Christie et al. (5, 6), and the mass spectrometric behavior by McCloskey and Law (9). Fatty acids with cyclopropane hydrocarbon end groups are, however, very rare. 9-Cyclopropanenonanoic acid appears to be the only such acid reported in the literature.

9-Cyclopropanenonanoic acid. This acid was prepared from undec-10-enoic acid essentially as described by Christie et al. (5, 6). Methyl 9-cyclopropanoate (9.0 g) at a boiling point of 112 to 114 C (1 mm; purity, 98%, GLC) was obtained from 36.9 g (0.186 mol) of methyl undec-10-enoate. The free acid was crystallized from acetone at −20 C (melting point, 39.8 to 40.5 C).

12-Cyclopropanedodecanoic acid. Undec-10-enoic acid (51.5 g [0.280 mol]) and 41.0 g (0.280 mol) of methyl hydrogen pentan-1,5-dioate were electrolyzed (8). The reaction product was distilled and methyl tetradec-13-enoate (20.8 g), boiling at 140 to 150 C (1 mm), was collected. Methyl 12-cyclopropanedodecanoate (5.5 g; boiling point, 175 to 180 C, 2 mm) was prepared from 20.8 g of the unsaturated methyl ester as described. Hydrolysis afforded the free acid which was crystallized from acetone in the cold (melting point, 54.2 to 55.2 C).

17-Cyclopropaneheptadecanoic acid. 9-Cyclopropanenonanoic acid (1.5 g [0.0076 mol]) and 3.3 g (0.0152 mol) of methyl hydrogen decan-1,10-dioate were electrolyzed (8). The crude methyl 17-cyclopropaneheptadecanoate was purified by chromatography on 100 g of silicic acid (Mallinckrodt, 100 mesh) with ether-light petroleum (boiling point, 40 to 60 C) (1:20 vol/vol) as eluant. An ester (858 mg) of satisfactory purity (98% by GLC) was obtained. Hydrolysis afforded 720 mg of the free acid which was crystallized from acetone (melting point, 69.4 to 70.2 C).

Activity measurements and surface film technique. A detailed description of these experimental procedures was given in the earlier paper of the series (see reference 8).

RESULTS AND DISCUSSION

The biological activity, mainly with regard to the test organism *F. roseum*, and the collapse properties of the surface films formed on water will be described below. The surface film behavior was discussed in our previous paper. Thus, there is usually a strong tendency to form a triple molecular layer, and the instability of the condensed monolayer at compression to small molecular cross-section areas is shown by the reduction in pressure after the collapse point, or when the compression is stopped until equilibrium is reached. In the previous paper it was suggested that there is a relation between the biological activity and the degree of instability of the monolayer, expressed by the reduction in pressure when the triple layer is formed from the monomolecular layer.

Neo-acids. The inhibitory effect of acids of *neo*-configuration on the germination of conidia of *F. roseum* was tested as described earlier (8). 12-Methyltridecanoic acid, the behavior of which is known from previous work, was used for comparison. In the experiments, 12,12-dimethyltridecanoic acid in amounts of 50, 100, and 200 μg per ml of nutrient solution and 18,18-dimethylnonadecanoic acid (100 and 200 μg/ml) were used alone and in combination with the fungicide tetramethylthiuramdisulfide (TMTD) (0.5 μ/ml). The results, summarized in Fig. 1 and Table 1, indicate that the short *neo*-fatty acid behaves very similarly to the fatty acid of *iso*-configuration, although the former, when used alone in the concentration of 100 μg/ml, produces somewhat lower inhibition indices after 3 h of incubation. However, in both cases a prolonged incubation reduces the effect considerably. One reason for the reduced effect in the case of longer incubation time might be that these compounds only have fungistatic effect in concentrations, so that the difference in inhibition illustrates the growth during the prolonged incubation time. Furthermore, 12,12-dimethyltridecanoic acid enhanced the inhibitory effect of TMTD as efficiently as did 12-methyltridecanoic acid. On the other hand, the long *neo*-fatty acid, 18,18-dimethylnonadecanoic acid, had hardly any effect when used alone, and only a moderate effect when used in combination with TMTD.

The surface film behavior of 12,12-dimethyl-

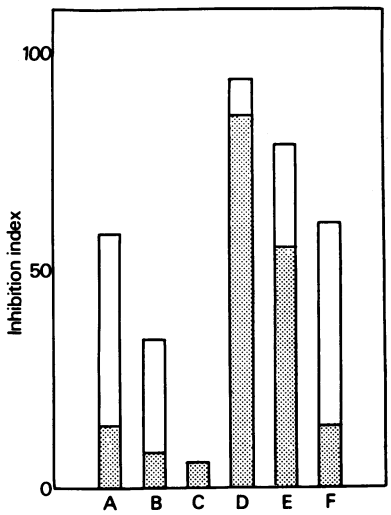

FIG. 1. *Inhibition of the germination of conidia of Fusarium roseum, incubated at 25 C in nutrient solution, supplemented with 100 μg of iso-, neo-, and cyclopropane fatty acids per ml. The filled portion of the columns represents the inhibition indices after 4 h of incubation, and the total height corresponds to 3 h of incubation. Relative number of germinating conidia in the nutrient solution is equal to 100. (A) 12-Methyltridecanoic acid; (B) 12,12-dimethyltridecanoic acid; (C) 18,18-dimethylnonadecanoic acid; (D) 9-cyclopropanenonanoic acid; (E) 12-cyclopropanedodecanoic acid; (F) 17-cyclopropaneheptadecanoic acid.*

tridecanoic acid is illustrated in Fig. 2. In the investigated temperature range of 5 to 35 C, it forms a liquid-condensed phase only. An unusual property of this liquid-condensed phase is that it does not show the usual type of sharp collapse point. The collapse starts at an area of about 32 to 31 Å² per molecule, and this is also a remarkably high value. Therefore, this compound should be expected to show an expansion effect on a lipid bilayer in which it is incorporated. The expansion effect on a monomolecular surface film of purified egg lecithin on water was studied (experimental conditions given in reference 8). Up to about 2 molecules of *neo*-acid per lecithin molecule can be dissolved in the liquid-condensed lecithin phase. The average area per hydrocarbon chain at the collapse point was plotted as a function of composition, and a nearly linear increase in hydrocarbon chain cross-section area resulted from 28.0 Å² in the pure lecithin film to 29.5 Å² at a molecular lecithin/*neo*-acid ration of 2:1. This short *neo*-acid shows a strong synergistic effect in combination with TMTD (Table 1).

18,18-Dimethylnonadecanoic acid shows, as does the shorter homologue, only one monolayer phase in the temperature range of 5 to 35 C. This phase is also of liquid-condensed type, but the collapse point of the monolayer is better defined, and it has a somewhat smaller value (30.2 Å²/molecule at 20 C). There is a considera-

TABLE 1. *Synergistic effect of TMTD and the lipid components according to the inhibition of the germination of Fusarium roseum conidia*[a]

Lipid component added to the nutrient solution	Amount (μg/ml)	Inhibition index			Synergistic effect
		Lipid components alone	TMTD (0.5 μg/ml)	Lipid plus TMTD (0.5 μg/ml)	
12-Methyltridecanoic acid	100	14	9	60	++
12,12-Dimethyltridecanoic acid	50	6	8	43	
	100	8	8	58	++
	200	8	8	75	
18,18-Dimethylnonadecanoic acid	100	6	14	37	+
	200	6			
9-Cyclopropanenonanoic acid	25	14	9	29	
	50	31	9	79	++
	100	85	14	99	
	200	99			
12-Cyclopropanedodecanoic acid	25	20	9	13	
	50	26	9	35	
	100	55	14	95	+
	200	82			
17-Cyclopropaneheptadecanoic acid	25	11			
	50	12	9	15	(+)
	100	14	14	37	
	200	27			

[a] The incubation was for 4 h at 25 C. The relative number of germinating conidia in the nutrient solution is equal to 100.

ble decrease with increasing temperature in the collapse pressure and the equilibrium pressure at the monolayer-to-triple layer transition, which can be seen in Fig. 3. There is also a slight reduction in pressure after collapse at 20 C. This *neo*-acid shows a moderate synergistic effect (Table 1). The results given here and in the previous paper are interpreted as indications of two types of surface film behavior which can simulate the permeability effect on the plasma membrane. The first is the collapse behavior of the film in the liquid-condensed state (as the hydrocarbon chains in biomembranes). A reduction in pressure indicates that the two-dimensional phase is unstable, and a tendency to form three-dimensional aggregates therefore might be expected even in a membrane environment. The second is based on the expansion effect on a monomolecular film of a membrane lipid, and an increase in permeability should be expected in an expanded lipid film.

Cyclopropane acids. The same biological activity measurements were performed on the series of ω-cyclopropane fatty acids. Their effect in relation to the *iso*- and *neo*-fatty acids is illustrated in Fig. 1, and the effect as a function of concentration can be seen from Fig. 4. Clearly these acids are considerably more inhibitory than the *neo*-fatty acids of the corresponding chain length. In fact, 9-cyclopropanenonanoic acid in concentrations above 100 μg/ml functions like a fungicidal agent. Thus, in a nutrient solution containing 200 μg of this fatty acid per ml, only a small number of conidia germinated; even after 6 h of incubation the inhibition index was 82, and after 24 h of incubation few diminutive rosettes of abnormally thick hyphae were produced (Fig. 5 C). Furthermore, in growth experiments carried out in a nutrient solution containing 100 μg/ml, no measurable growth occurred within 11 days. On the other hand, the two longer cyclopropane fatty acids used in the same concentration merely caused a prolonged lag phase, as did 12-methyltridecanoic acid. However, abnormally thick hyphae were also formed in the presence of 12-cyclopropanedodecanoic acid, 200 μg/ml, but in this case

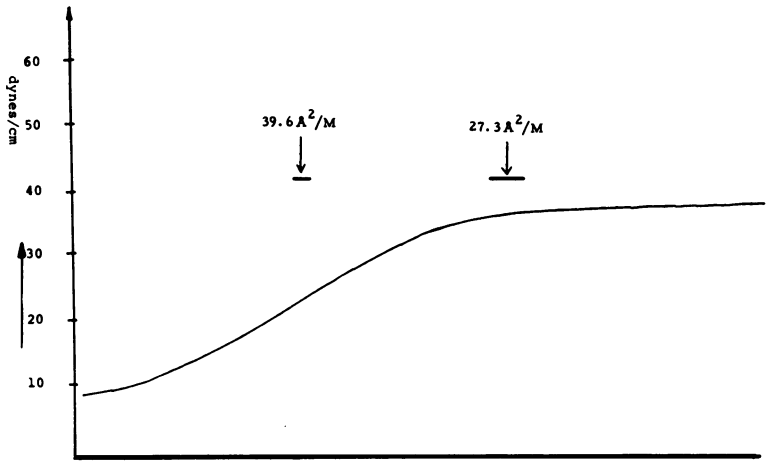

FIG. 2. *π-A isotherm of 12,12-dimethyltridecanoic acid at 20 C. The molecular area is given in linear form along the x axis by the values in two calibration points.*

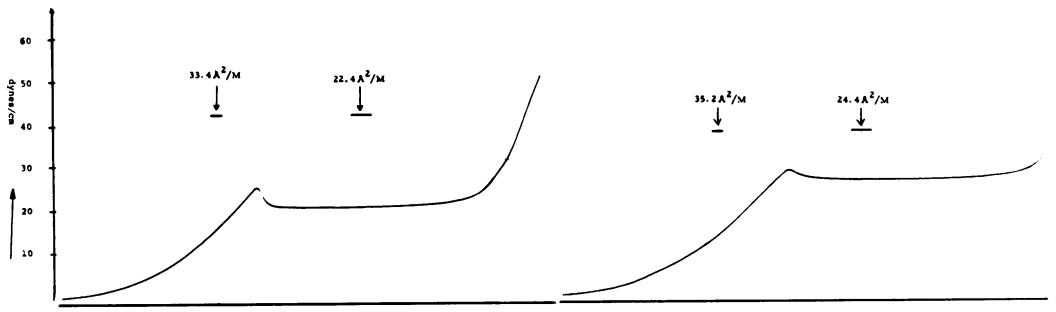

FIG. 3. *π-A isotherm of 18,18-dimethylnonadecanoic acid at 6 C (A) and at 21 C (B).*

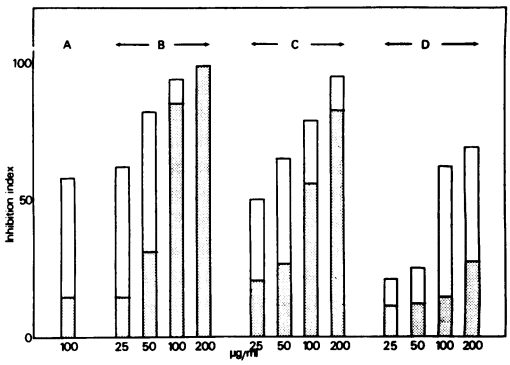

FIG. 4. *Inhibition of the germination of conidia of Fusarium roseum, incubated at 25 C in nutrient solution, supplemented with various amounts of cyclopropane fatty acids. The total columns represent the inhibition indices after 3 h and their filled portion represents the inhibition indices after 4 h of incubation. Relative number of germinating conidia in the nutrient solution is equal to 100. (A) 12-Methyltridecanoic acid; (B) 9-cyclopropanenonanoic acid; (C) 12-cyclopropanedodecanoic acid; (D) 17-cyclopropaneheptodecanoic acid.*

the distortion was overcome and apparently normal hyphae emerged from the abnormal strains (Fig. 5 D). The effect of the longest cyclopropane fatty acid tested was still less pronounced and only very few thick hyphae appeared (Fig. 5 E). The effect in combination with TMTD is shown in Table 1. All acids give an enhancement of the effect of TMTD which is higher than their effect when used alone. Obviously, the best synergistic effect is produced by 9-cyclopropanenonanoic acid. 9-Cyclopropanenonanoic acid does not show a condensed monomolecular phase on water, as the disordering effect in the water surface prevents condensation of such short chains. The possibility of studying the expansion effect in surface film phases therefore is limited. Efforts to solubilize this acid in monomolecular films of lecithin were unsuccessful.

12-Cyclopropanedodecanoic acid shows a solid-condensed monolayer phase below 10 C, with a collapse area of about 23.9 Å²/molecule. Above this temperature there is only a liquid-condensed monolayer phase with a very low collapse pressure, as can be seen in Fig. 6. The molecular area at collapse of about 28 Å²/molecule at room temperature is in good agreement with that of *iso*-tetradecanoic acid, and there is also a similar reduction in pressure after collapse of this phase. The pressure reduction after collapse is about 5 and 2.5 dynes/cm for the *iso*-fatty acid and ω-cyclopropane fatty acid, respectively (see Fig. 6 and reference 8). The synergistic effects of these two acids with TMTD are as shown in Table 1.

The surface film behavior of 17-cyclopropaneheptadecanoic acid was examined in the range of 5 to 35 C. A very remarkable feature is that it forms solid-condensed phases with vertical chains over the whole temperature range. They are in this respect similar to normal fatty acids, and this illustrates the significance of the size of the end group. Although an *iso*-methyl end group is not much larger, these acids do not form phases with vertical molecules. The π-A isotherm (Fig. 7), corresponding to 20 C, shows that there is no liquid-condensed phase. A solid-condensed phase, which according to viscosity properties and molecular area seems to be an LS-phase, is formed from the gas phase, and at 19.4 Å²/molecule there is a first-order transition into a CS-phase. At temperatures above 30 C the CS-phase is no longer formed, and collapse of the monolayer starts at about 19.5 Å²/molecule. Although there is a considerable reduction in pressure after collapse of the monolayer, there is still a stable monolayer phase which is quite condensed (LS). This might explain why there is no significant synergistic effect with TMTD (Table 1), as the earlier reported relation between post-collapse reduction in pressure and synergistic effect with TMTD was only observed when the lipid exhibited a liquid-condensed phase (8).

Of the two homologue series studied, the strongest antimicrobial effect is obtained from 9-cyclopropanenonanoic acid. When used alone the effect is in fact so high that it can be classified as a fungicide and drastic morphological changes of the test organisms are obtained (Fig. 5).

The effects of these lipids on gram-positive and gram-negative bacteria were also studied using *Staphylococcus aureus* and *Escherichia coli*. As in the previous study it was found that only the gram-positive organism is inhibited, and the various lipids possess the same relative effect as against the fungus *F. roseum*. The minimal inhibitory concentrations against *S. aureus* are (methanol was used as solvent): 9-cyclopropanenonanoic acid, 500 μg/ml; 12-cyclopropanedodecanoic acid, 500 μg/ml; 17-cyclopropaneheptadecanoic acid, >500 μg/ml; 12,12-dimethyltridecanoic acid, 100 μg/ml (which can be compared with 12-methyltridecanoic acid, 100 μg/ml); and TMTD (dissolved in polyethylene glycol), 10 μg/ml. The most interesting compound with regard to pharmacological applications is 9-cyclopropanenonanoic acid, which also has been tested against fungi other than *F. roseum*. The minimal inhibitory concentration

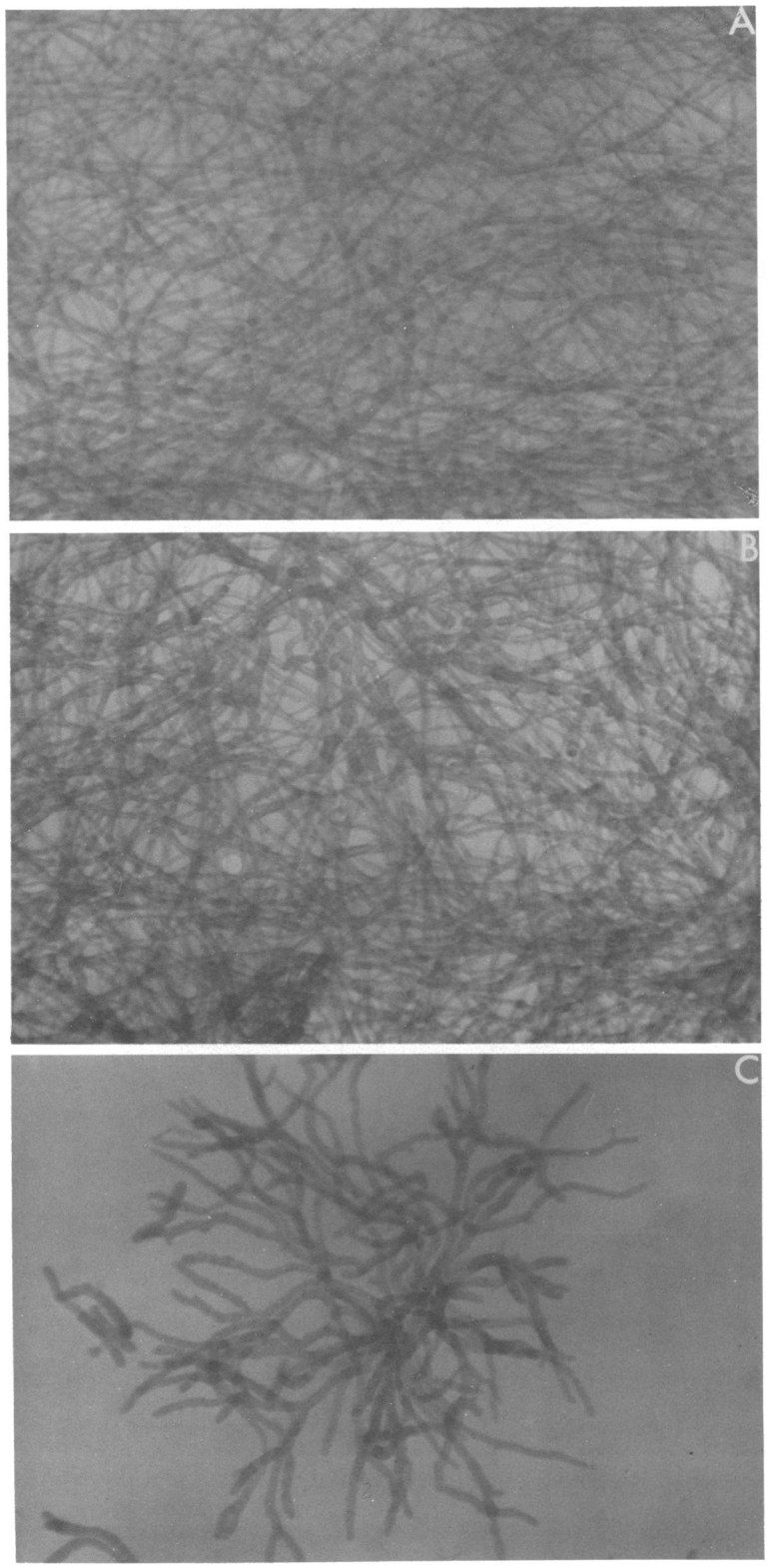

FIG. 5. *Micelles formed from germinating conidia, incubated for 24 h in nutrient solution supplemented with various fatty acids in a concentration of 200 μg/ml. Magnification × 320. (A) No supplement (control). Normal hyphae. (B) 12-Tridecanoic acid. A dense micelle with somewhat thicker hyphae than in the control. (C) 9-Cyclopropanenonanoic acid. Few diminutive rosettes with abnormally thick hyphae. (D) 12-Cyclopropanedodecanoic acid. Micelle with thick hyphae, from which normal hyphae are emerging. (E) 17-Cyclopropaneheptadecanoic acid. Loose micelle with single strands of thick hyphae.*

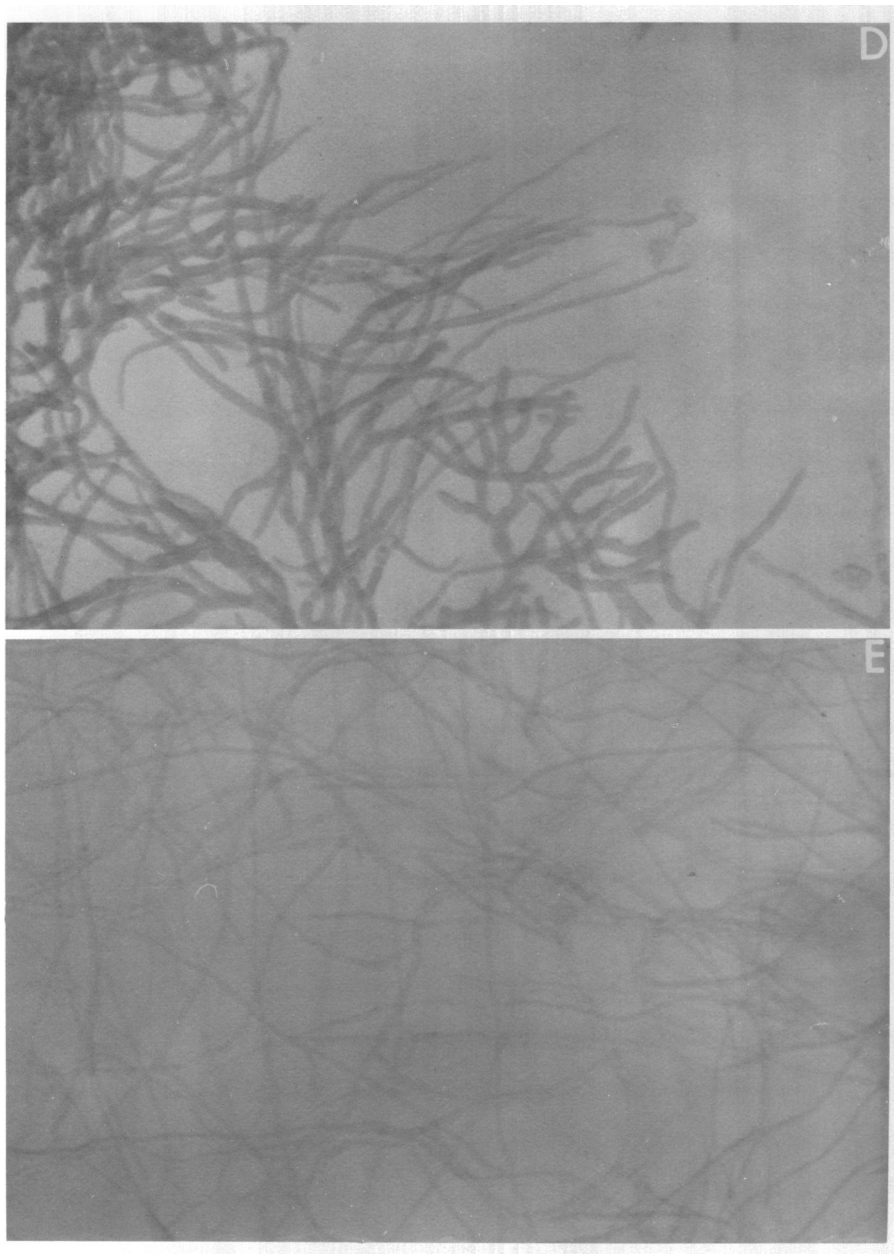

FIG. 2—*Continued*

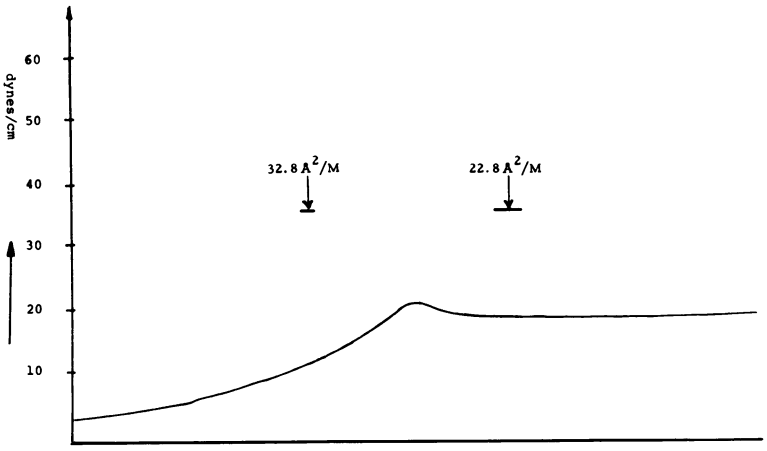

FIG. 6. *π-A isotherm of 12-cyclopropanedodecanoic acid at 20 C.*

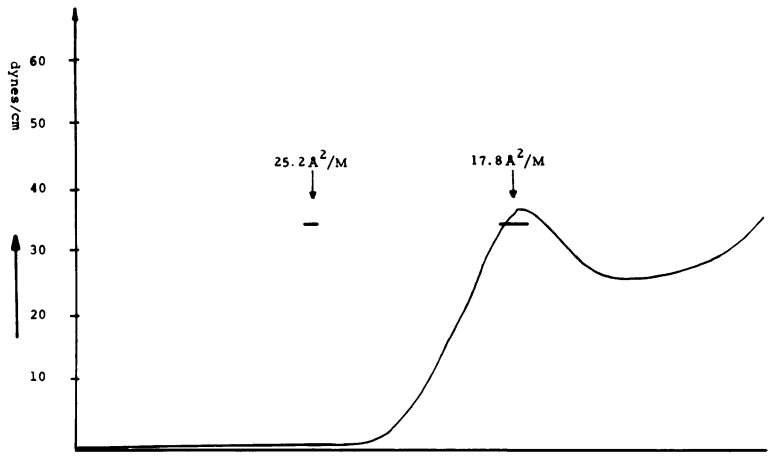

FIG. 7. *π-A isotherm of 17-cyclopropanedodecanoic acid at 20 C.*

values against *Candida albicans* and *Aspergillus niger* are both 500 μg/ml, whereas the corresponding values obtained by 12-methyltridecanoic acid are >1,000 and 1,000 μg/ml, and by TMTD, 5 and 50 μg/ml, respectively.

Polyene antibiotics have been extensively studied (see reference 7), and it has been shown that they act by changing the membrane permeability. They have effect only when the membrane contains sterols, and recent studies indicate that they form a stoichiometric complex with cholesterol (11). The effect of the fatty acids examined in the present work on the membrane permeability cannot be due to the same kind of interactions. This is evident from the fact that they give the same effect against gram-positive bacteria as against fungi, although the former organisms have no cholesterol in their plasma membrane.

ACKNOWLEDGMENTS

Grants from the Swedish Board for Technical Development and the Swedish Natural Science Research Council to support this effort are gratefully acknowledged.

LITERATURE CITED

1. Arosenius, K. E., S. Ställberg-Stenhagen, and E. Stenhagen. 1949. Branched long chain compounds. The synthesis of 20,20-dimethylheneicosanoic and 14,14-dimethylpentadecanoic acids. Ark. Kemi 1:413–418.
2. Baer, E., and H. O. L. Fischer. 1945. Synthesis of a homologous series of optically active normal aliphatic α-monoglycerides (L-series). J. Am. Chem. Soc. 67:2031–2037.
3. Barton, D. H. R., and E. P. Serebryakov. 1962. A convenient procedure for the decarboxylation of acids. Proc. Chem. Soc. Sept., p. 309.
4. Carter, F. L., and V. L. Frampton. 1964. Review of the chemistry of cyclopropene compounds. Chem. Rev. 64:497–525.
5. Christie, W. W., F. D. Gunstone, I. A. Ismail, and L. Wade. 1968. The synthesis and chromatographic and

spectroscopic properties of the cyclopropane esters derived from all the methyl octadecenoates (Δ^2-Δ^{17}). Chem. Phys. Lipids 2:196–202.
6. Christie, W. W., and R. T. Holman. 1966. Mass spectrometry of lipids. I. Cyclopropane fatty acid esters. Lipids 1:176–182.
7. Kinsky, S. C. 1970. Antibiotic interaction with model membranes. Annu. Rev. Pharmacol. 10:119–100.
8. Larsson, K., B. Norén, and G. Odham. 1972. Antimicrobial effect of simple lipids and its relation to surface film behavior. Biochem. Pharmacol. 21:947–962.
9. McCloskey, J. A., and J. H. Law. 1967. Ring location in cyclopropane fatty acid esters on long-chain acids. Lipids 2:225–200.
10. Meyer, H., and G. C. Holz, Jr. 1966. Biosynthesis of lipids by kinetoplastic flagellates. J. Biol. Chem. 241:5000–5007.
11. Norman, A. W., R. A. Demel, B. deKruyff, and L. L. M. van Deenen. 1972. Studies on the biological properties of polyene antibiotics. Evidence for the direct interaction of filipin with cholesterol. J. Biol. Chem. 247:1918–1929.
12. Odham, G. 1965. The chemical composition of the wax in the free flowing secretion from the preen gland of the mute swan (Cygnus olor). Ark. Kemi 23:431–451.
13. O'Leary, W. M. 1962. The fatty acids of bacteria. Bacteriol. Rev. 26:421–447.
14. Smith, R. D., and H. E. Simmons. 1961. Norcarane (Bicyclo [4.1.0] heptane). Org. Synth. 41:72–75.
15. Sobotka, H., and F. E. Stynler. 1950. Neo-fatty acids. J. Am. Chem. Soc. 72:5139–5143.