

groups negatively charge the packing surface. Consequently, BzAC cations were electrostatically bonded on the packing surface and not eluted.

It was observed that the k' values of BzAC decreased greatly by increasing the concentration of NaCl, but increased when the concentration exceeded 0.05 M/L. This phenomenon may be caused by a combination of ion suppression and salting out effects on the addition of NaCl.

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REFERENCES

1. Nakae, A., and K. Kunihiro, *J. Chromatogr.* 134:459 (1977), 152:137 (1978), and 156:167 (1978).
2. Parris, N., W.M. Linfield and R.A. Barford, *Anal. Chem.* 49:2228 (1977).
3. Sakamoto, K., M. Takehara and A. Ishiwata, 32nd annual

- meeting of colloid and surface chemistry, Kochi-shi, Japan, Oct. 1979, p. 264.
4. Nakamura, K., Y. Morikawa and I. Matsumoto, *Yukagaku* 29:501 (1980).
 5. Karger, B.L., in "Modern Practice of Liquid Chromatography," edited by J.J. Kirkland, Wiley-Interscience, New York (1971), p. 10.
 6. Uno, T., K. Miyajima and T. Nakagawa, *Bunseki Kagaku* 15:584 (1966).
 7. Horvath, C., W. Melander and I. Molanar, *J. Chromatogr.* 125:129 (1976).
 8. Horvath, C., W. Melander and I. Molanar, *Anal. Chem.* 49:142 (1977).
 9. Horvath, C., and W. Melander, *J. Chromatogr. Sci.* 15:393 (1977).
 10. Nakamura, K., Y. Morikawa and I. Matsumoto, *Bunseki Kagaku* 29:314 (1980).
 11. Scholfield, C.R., *JAOCs* 52:36 (1975).
 12. Plattner, R.D., G.F. Spencer and R. Kleiman, *Ibid.* 54:511 (1977).
 13. Scott, R.P.W., and P. Kucera, *J. Chromatogr.* 142:213 (1977).
 14. Hanai, T., and K. Fujimura, *J. Chromatogr. Sci.* 14:140 (1976).

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Surface Active Properties of a Biosurfactant from *Corynebacterium lepus*

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ABSTRACT

A mixture of corynomycolic acids ($R^1-CH(OH)-CH(R^2)-COOH$) isolated from *Corynebacterium lepus* was shown to have excellent surfactant properties. It caused significant lowering of surface tension in aqueous solution and the interfacial tension between water and hexadecane at all values of pH between 2 and 10. A series of carboxylic acids and some hydroxy-carboxylic acids and alcohols were also studied as a comparison. None of these caused as large a lowering of the surface and interfacial tensions as the corynomycolic acids. The series of carboxylic acids studied showed that surfactant properties depend on the length of the alkyl chain and the pH of the solution in a manner consistent with the hydrophilic-lipophilic balance of these compounds. Hydroxyl substituents caused considerable enhancement of the surfactant properties of long chain carboxylic acids if they were located close to the carboxyl function.

INTRODUCTION

Corynebacterium lepus grown on kerosene produces a mixture of surface active agents (1). One of the major components in this mixture is a class of β -hydroxy- α -branched carboxylic acids (2) called corynomycolic acids (3,4). These acids, present in the *C. lepus* whole broth, have been shown to be effective agents to enhance bitumen recovery by a cold water extraction process (5,6). For this reason a study was undertaken of the surfactant properties of these acids as determined by surface and interfacial tension.

Spreading pressures of many carboxylic acids have been reported (7-13) as well as some determinations of the effect of pH changes (7,9,12,13). The spreading pressures of

several hydroxy-carboxylic acids have also been measured (7,14-16).

This work considered the surfactant properties of the mixture of corynomycolic acids isolated from *C. lepus*. These were evaluated by measuring the surface tension and interfacial tension against hexadecane of saturated aqueous solutions over a wide range of pH. In an attempt to correlate the importance of structural features of the corynomycolic acids to their observed surface properties, similar studies were made of saturated carboxylic acids, hydroxy-carboxylic acids and alcohols.

MATERIALS AND METHODS

Materials

Most of the chemicals, including all of the saturated unsubstituted fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO). The 16-hydroxy-hexadecanoic acid and hexadecanol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Triaccontanol was purchased from Polyscience Corp. (Evanston, IL). The 2-hydroxy-stearic acid was purchased from Pfaltz and Bauer Inc. (Flushing, NY).

The hexadecane was first cleaned by washing alternately with sodium hydroxide (4 N) and concentrated sulfuric acid and then distilled water until an interfacial tension with water of 45 dyne/cm was obtained.

Production, Isolation and Characterization of Corynomycolic Acids

The production, isolation and characterization of the corynomycolic acids ($R^1-CH(OH)-CH(R^2)-COOH$) from *C. lepus* are reported in full in other publications (1,2) and are only briefly summarized here. The organism was grown on

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kerosene and the floating white product collected by suction and lyophilized. The corynomycolic acids were isolated by saponification, converted to the methyl esters, chromatographed on an alumina column and resaponified. This mixture of corynomycolic acids was characterized by standard methods, including infrared spectroscopy and mass spectrometry (2). The R^1 chain contained from 16 to 25 carbon atoms and the R^2 chain contained from 8 to 13 carbon atoms (2).

Surface and Interfacial Tension Determination

Surface tension and interfacial tension were measured with a de Nouy surface tensiometer (Fisher Autotensiomat) at 20 C (± 1 C). Unless otherwise noted, 0.5 mg of carboxylic acid was used per ml of water. This was sufficient to give a saturated solution with the most soluble acids (17). Since the surface tension of a saturated solution is not substantially altered if an excess of a carboxylic acid is used (13), this method allowed a comparison of the surfactant properties of different acids at their critical micelle concentrations (18). The pH of these mixtures was adjusted with either sodium hydroxide or hydrochloric acid. Nutting and Long studied the dynamics of the surface tension of lauric acid over a range of pH values and reported that if the amount of the acid added was larger than the critical micelle concentration, equilibrium was achieved very quickly (13). For each sample several measurements of surface tension and pH were made until equilibrium was reached and constant values obtained. For the least soluble acids, up to 24 hr were necessary to reach equilibrium, but most took less than 1 hr. When the above measurements indicated that equilibrium had been reached, the interfacial tension against hexadecane was determined. Initially, these measurements were obtained in two ways. The measurement could be made immediately after layering hexadecane on the aqueous phase (18) or after vigorous agitation of the two phases to allow partitioning of the fatty acids. Data obtained for tetradecanoic acid by these methods over a wide pH range were identical and 0.5 g/l was sufficient to saturate both phases in each case. Therefore, the first method was used to determine interfacial tension for the rest of the compounds.

RESULTS

Corynomycolic Acids

Figure 1 contains plots of the surface and interfacial tensions of the mixture of corynomycolic acids from *C. lepus* vs the weight of acid present for two different values of pH. None of these curves exhibited a well-defined critical micelle concentration. There was a decrease in all the measurements with increasing concentration until about 0.3 mg/ml. The surface tension and interfacial tension measurements were both relatively insensitive to pH changes. This is further demonstrated in Figure 2. The plots of surface tension and interfacial tension of the corynomycolic acid mixture (0.5 g/l) indicate that these parameters are almost independent of pH, although both show a slight decrease with increasing pH. Figure 2 also contains data for the methyl esters of the corynomycolic acids. At every pH, the values of both surface tension and interfacial tension were lower for the acids than for the methyl esters.

Normal Alkyl Carboxylic Acids

Surface and interfacial tensions were measured for a range of saturated fatty acids at several values of pH between 2 and 10. Both the surface and interfacial tensions showed obvious correlations with the length of the alkyl chains. This is illustrated by Figures 3 and 4, which are plots of

surface tension and interfacial tension vs the number of carbon atoms in the acids at pH 9.0 and 4.0. The general trend is the same at other pH values. The saturated aqueous solutions of the longest carboxylic acids have surface and interfacial tensions similar to distilled water. As the alkyl chain becomes shorter, these values decrease to a minimum value at between ten to fifteen carbon atoms. The position of the minimum surface and interfacial tensions was affected by pH. The minimum surface tension for all these curves is between 35 and 40 dyne/cm; however, the minimum is at undecanoic acid at pH 4.0, at dodecanoic acid at pH 7.0 and at tetradecanoic acid at pH 9.0.

Hydroxy-Carboxylic Acids

Figure 5 illustrates the effect of a hydroxyl substituent on the surfactant properties of octadecanoic acid. The unsub-

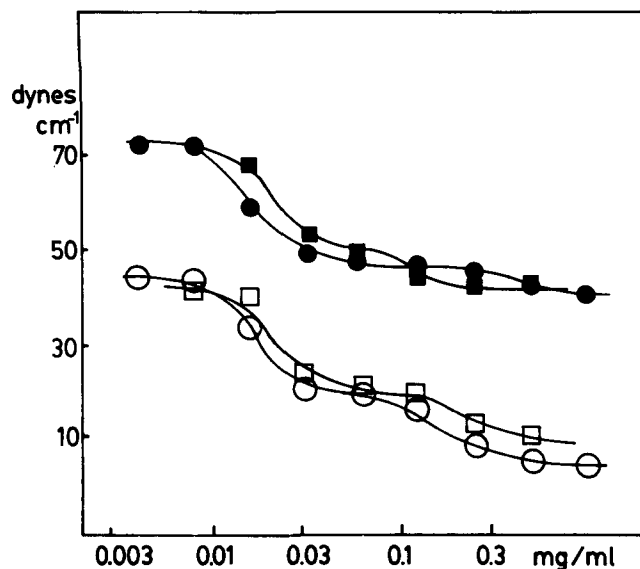


FIG. 1. The surface tensions at pH 5.5 (●) and pH 8.5 (■) and the interfacial tensions against hexadecane at pH 5.5 (○) and pH 8.5 (□) for different amounts of the mixture of corynomycolic acids plotted on a log scale.

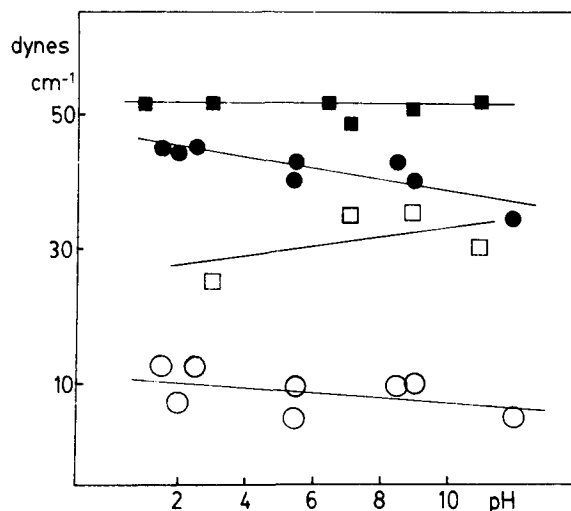


FIG. 2. The surface tension (●) and interfacial tension against hexadecane (○) vs pH of the aqueous solution of the mixture of corynomycolic acids (0.5 mg/ml). Also, the surface tension (■) and interfacial tension (□) of the mixture of methyl corynomycolates (0.5 mg/ml) vs pH.

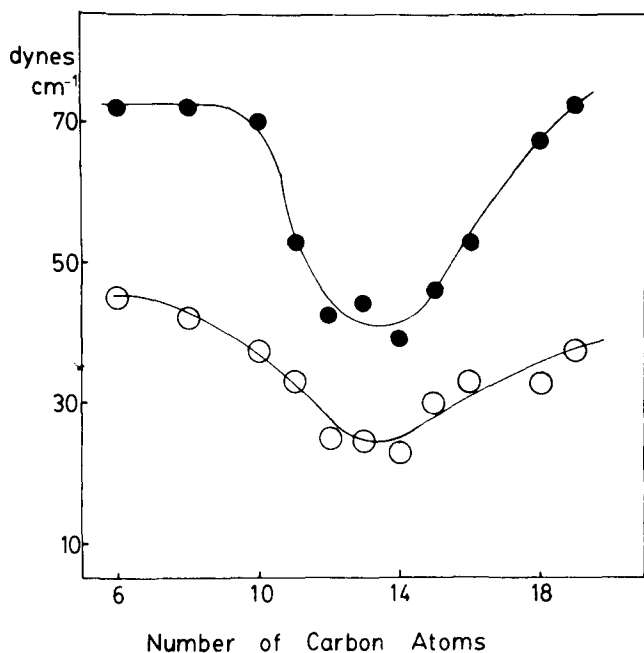


FIG. 3. Surface tension (●) and interfacial tension against hexadecane (○) of saturated solutions of carboxylic acids (0.5 mg/ml) vs the number of carbon atoms in each acid, at pH 9.0

stituted octadecanoic acid causes only a small lowering of the surface and interfacial tensions throughout the pH range, although it is a measurably better surfactant at higher pH. With a hydroxyl substituent on the twelfth carbon, there is an increased lowering of both parameters at every pH. However, when the hydroxyl substituent is adjacent to the carboxyl function, the acid becomes an excellent surfactant. Furthermore, the values of the 2-hydroxy-octadecanoic acid solutions are relatively insensitive to pH.

Similar behavior was observed for a comparison of hexadecanoic acid and hydroxy-hexadecanoic acids. The 2-hydroxy-hexadecanoic acid was an effective surfactant, but the hexadecanoic and 16-hydroxy-hexadecanoic were very poor.

Alcohols

Only a limited study was made of the surface properties of alcohols. The data for the three studied were found to be independent of pH. Table 1 lists the surface and interfacial tension at pH 7.0. The shortest alcohol, 2-octanol, showed no significant surfactant properties. The longer alcohols did cause some lowering of the interfacial tension, although the lowest value was only 33 dyne/cm. The effect on surface tension was more pronounced and hexadecanol caused a marked decrease in this parameter.

DISCUSSION

The mixture of corynomycolic acids isolated from *C. lepus* had good surfactant properties. Saturated, aqueous solutions of the mixture had surface tensions of about 45 dyne/cm and interfacial tensions of about 10 dyne/cm over the whole range of pH studied (Fig. 2). These results are impressive when compared to the data for unsubstituted, saturated, carboxylic acids. Although the minimum surface tensions caused by these simple acids were comparable to those seen for the corynomycolic acids, they did not lower the interfacial tension below 20 dyne/cm. Furthermore, the surface and interfacial tensions of the simple acids were

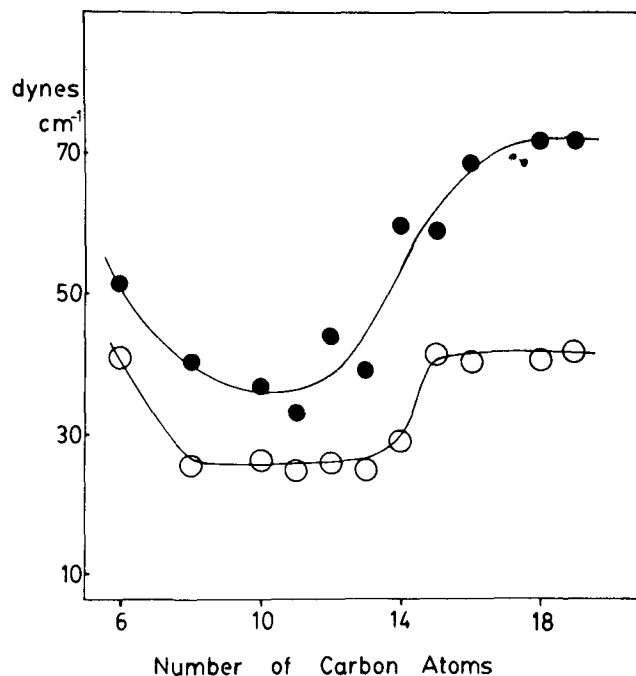


FIG. 4. Surface tension (●) and interfacial tension against hexadecane (○) of saturated solutions of carboxylic acids (0.5 mg/ml) vs the number of carbon atoms in each acid, at pH 4.0.

very sensitive to pH (7,9,12,13) and were effective surfactants within only small ranges. Finally, these acids were sensitive to the length of the alkyl chains (Fig. 3 and 4) and the carboxylic acids with more than 18 carbon atoms were very poor surfactants. The corynomycolic acids in the mixture from *C. lepus* were appreciably larger and all had more than twenty-five carbon atoms. These were partitioned between two alkyl branches (i.e., $R^1\text{-CH(OH)-CH(R}^2\text{)-COOH}$). However, the longer chain (R^1) contained twenty or more carbon atoms.

Thus, the corynomycolic acids are much more effective surfactants than would be predicted from the data for the simple carboxylic acids. The most reasonable explanation is an enhancement by the hydroxyl function on the carbon β to the carboxylic group of the corynomycolic acids. The study on the effect of adding a hydroxyl group to a carboxylic acid supported this conclusion. For example, octadecanoic acid was a relatively poor surfactant over the whole range of pH studied (Fig. 5). Both the hydroxy-octadecanoic acids studied were more effective surfactants; however, the position of the substitution was important. The 2-hydroxy-octadecanoic acid was an excellent surfactant and caused a lowering of surface and interfacial tensions, almost as large as the corynomycolic acids, for the whole pH range studied, but 12-hydroxy-octadecanoic acid was less effective. Similar results were obtained with a series of hexadecanoic acids.

The data for the long chain alcohols demonstrated that the hydroxyl function alone was a reasonable surfactant (Table I). These alcohols caused a lowering of surface and interfacial tensions although none were as effective as the best carboxylic acids.

When the carboxylic functions of the corynomycolic acid mixture were converted to methyl esters, the effectiveness of this surfactant decreased. The more polar carboxylic function resulted in a better surfactant than the ester.

Hydrophilic-lipophilic balance (HLB) can be used to characterize surfactants (19). A consideration of relative HLB within series of surfactants provided a reasonable

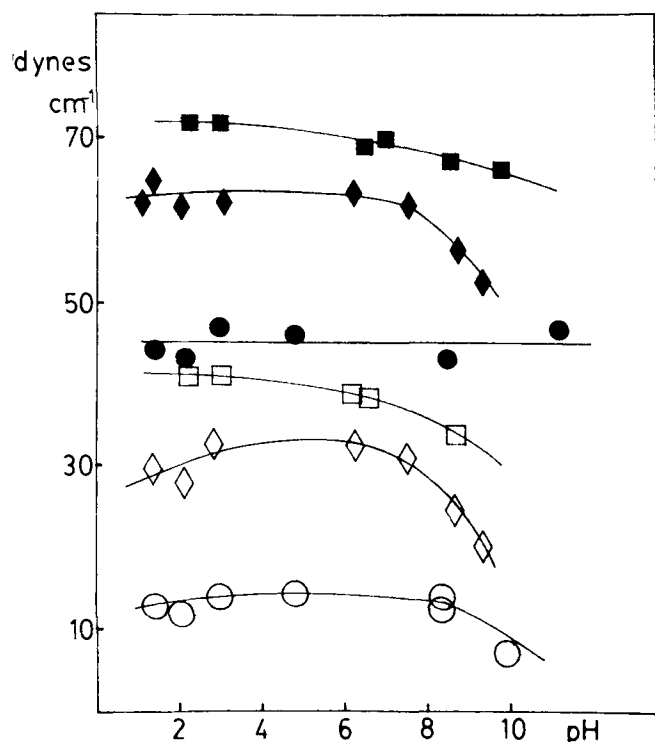


FIG. 5. Data for saturated solutions (0.5 mg/ml) of three carboxylic acids vs pH: octadecanoic acid, surface tension (■) and interfacial tension (□); 12-hydroxy-octadecanoic acid, surface tension (◆) and interfacial tension (◇); and 2-hydroxy-octadecanoic acid, surface tension (●) and interfacial tension (○).

explanation of many of the above observations. For example, the number of carbon atoms in a carboxylic acid will influence the HLB. The longer fatty acids are relatively lipophilic and the shorter acids more hydrophilic. Carboxylic acids with an intermediate chain length of ten to fifteen carbon atoms were the best surfactants, indicating a favorable balance. As the acids became longer and more lipophilic they were poorer surfactants. Similarly, the changes caused by pH can be attributed to changes in the relative concentrations of the neutral carboxylic acids and the more hydrophilic carboxylate ions formed at higher pH. The longer fatty acids became better surfactants at high pH because their carboxylate ions had a more favorable balance with the lipophilic alkyl chain.

These considerations also explain the effect of the hydroxyl function on the surfactant abilities of carboxylic acids. A hydroxyl function close to the carboxyl group of a long fatty acid or corynomycolic acid renders it more hydrophilic, resulting in a more favorable HLB with the large alkyl chains. If the hydroxyl substituent is isolated from the carboxylic function, the effect is absent.

The corynomycolic acids will have an unusual orientation at the aqueous interface because each molecule will have two hydrophilic functional groups in the water and two hydrophobic chains in the upper oil or air phase. This is analogous to the surface packing for some phospholipids (20,21) and synthetic surfactants such as α -sulpho-branched chain fatty acids (22) which are known to have good surfactant properties. The β -hydroxy function of the corynomycolic acid is in the proper position to form a stable, 6-membered, hydrogen bonded ring with the carboxylic group (23). Such an interaction would influence

TABLE I

The Surface and Interfacial Tensions (dyne/cm) of Saturated Aqueous Solutions of Several Alcohols (0.5 mg/ml, pH 7.0, 25 C)

	Surface tension	Interfacial tension
2-octanol	68	46
1-hexadecanol	42	36
1-triacontanol	53	33

this biosurfactant's effectiveness and pH dependence. Another possibility is intermolecular hydrogen bonding between the hydroxyl function of one molecule and the carboxylic group of the adjacent acid. Intermolecular associations of dual-functional phospholipids have been proposed as a significant factor in their surfactant properties (24).

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REFERENCES

- Cooper, D.G., J.E. Zajic and D.F. Gerson, *Appl. Environ. Microbiol.*, 37:4 (1979).
- Cooper, D.G., J.E. Zajic and D.E.F. Gracey, *J. Bacteriol.*, 137:795 (1979).
- Asselineau, C., and J. Asselineau, *Bull. Soc. Chim. Fr.*, 1992 (1966).
- Welby-Gieusse, M., M.A. Laneelle and J. Asselineau, *Eur. J. Biochem.*, 13:164 (1970).
- Gerson, D.F., and J.E. Zajic, *Dev. Ind. Microbiol.*, 19, 577 (1978).
- Gerson, D.F., and J.E. Zajic, *The Oil Sands of Canada-Venezuela*, CIMM, 17:705 (1977).
- Adam, N.K., *The Physics and Chemistry of Surfaces*, 3rd ed. Oxford Univ. Press, London, 1941.
- Powney, J., and C.C. Addison, *Trans. Faraday Soc.*, 34:372 (1938).
- Lyons, C.G., and E.K. Rideal, *Proc. R. Soc. London*, 124A: 322 (1929).
- Roe, C.P., and P.D. Brass, *J. Amer. Chem. Soc.*, 76:4703 (1954).
- Campbell, A.N., and G.R. Lakshminarayanan, *Can. J. Chem.*, 43:1729 (1965).
- Goddard, E.D., *Croat. Chem. Acta*, 45:13 (1973).
- Nutting, G.C., and F.A. Long, *J. Amer. Chem. Soc.*, 63:84 (1941).
- Ries, H.E., and D.C. Walker, *J. Colloid Sci.*, 16:361 (1961).
- Wells, H.L., G. Zografis, C.M. Scrimgeour and F.D. Gunstone, *Adv. Chem. Ser. (ACS)*, 144:135 (1975).
- Kellner, B.M.J., and D.A. Cadenhead, *J. Colloid and Interface Sci.*, 63:452 (1978).
- Singleton, W.S., *Fatty Acids, Their Chemistry, Properties, Production and Uses*, part I, chapt. 7, p. 609 (1960).
- Cupples, H.L., *Ind. Eng. Chem.*, 29:924 (1937).
- Gerson, D.F., J.E. Zajic and M.D. Ouchi, *A.C. S. Symposium Series 90:66 (1979)*.
- van Deenen, L.L.M., U.M.T. Houtsmuller, G.H. deHaas and C. Mulder, *J. Pharm. Pharmacol.*, 14:429 (1962).
- Small, D.M., *JAACS* 45:108 (1968).
- Stirton, A.J., *Ibid.*, 39:490 (1962).
- Morgan, C.D., and N. Polyar, *J. Chem. Soc.*, 3779 (1957).
- Hayashi, M., T. Muramatsu, I. Hara and T. Seimiya, *Chem. Phys. Lipids*, 15:209 (1975).

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