THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL.
XI. THE CONSTITUTION OF NISINIC ACID $C_{24}H_{36}O_2$ IN SARDINE OIL.

By **Yoshiyuki TOYAMA** and **Tomotaro TSUCHIYA.**

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As stated in the 10th report of this series,⁽¹⁾ nisinic acid $C_{24}H_{36}O_2$ was separated as an individual component of highly unsaturated C_{24}-acids in sardine oil. In continuation to the previous study this paper deals with the experimental results obtained by the ozonolysis of amyl nisinate with a view to gain an insight into the constitution of nisinic acid.

On examining the products obtained by the ozonolysis of amyl nisinate, the following compounds were found: propyl aldehyde $CH_3{\cdot}CH_2{\cdot}COH$, propionic acid $CH_3{\cdot}CH_2{\cdot}COOH$, acetaldehyde $CH_3{\cdot}COH$, acetic acid $CH_3{\cdot}COOH$, carbon dioxide CO_2, succinic acid $HOOC{\cdot}(CH_2)_2{\cdot}COOH$ and amyl hydrogen succinate $HOOC{\cdot}(CH_2)_2{\cdot}COOC_5H_{11}$.

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(1) This Bulletin, **10** (1935), 543.

Of these compounds, propyl aldehyde and propionic acid are derived from the group $CH_3{\cdot}CH_2{\cdot}CH{=}$. Acetaldehyde and acetic acid together with carbon dioxide are attributable to the secondary decomposition of malonic acid and the corresponding aldehyde which are derived from the group $={CH}{\cdot}CH_2{\cdot}CH{=}$, while succinic acid and amyl hydrogen succinate are derived from the groups $={CH}{\cdot}(CH_2)_2{\cdot}CH{=}$ and $={CH}{\cdot}(CH_2)_2{\cdot}COOC_5H_{11}$ respectively. Accordingly, amyl nisinate was found to contain the following groups: $CH_3{\cdot}CH_2{\cdot}CH{=}$, $={CH}{\cdot}CH_2{\cdot}CH{=}$, $={CH}{\cdot}(CH_2)_2{\cdot}CH{=}$ and $={CH}{\cdot}(CH_2)_2{\cdot}COOC_5H_{11}$. It was also found from the yield of carbon dioxide obtained by the ozonolysis that amyl nisinate contained more than two of the group $={CH}{\cdot}CH_2{\cdot}CH{=}$. These results, coupled with the fact that nisinic acid consists of 24 carbon atoms, show that nisinic acid is composed of the following groups: $CH_3{\cdot}CH_2{\cdot}CH{=}$, three of $={CH}{\cdot}CH_2{\cdot}CH{=}$, two of $={CH}{\cdot}(CH_2)_2{\cdot}CH{=}$, and $={CH}{\cdot}(CH_2)_2{\cdot}COOH$. No evidence was brought out in these experiments regarding the respective positions of the groups $={CH}{\cdot}CH_2{\cdot}CH{=}$ and $={CH}{\cdot}(CH_2)_2{\cdot}CH{=}$. If, however, it is assumed that nisinic acid has ethylenic linkings in 4:5-, 8:9- and 12:13-positions as is the case with the previously studied moroctic acid $(\Delta^{4:5,\ 8:9,\ 12:13,\ 15:16})$,(2) eicosatetraenoic acid $(\Delta^{4:5,\ 8:9,\ 12:13,\ 16:17})$,(3) and clupanodonic acid $(\Delta^{4:5,\ 8:9,\ 12:13,\ 15:16,\ 19:20})$,(4) the constitution of nisinic acid should be represented by the following formula:

$$CH_3{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH \\ ={CH}{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}COOH.$$

Experimental.

1. **Separation of Nisinic Acid.** In previously reported experiments of the separation of hiragonic, moroctic and eicosatetraenoic acids, 60 kg. of the ethyl ester prepared from sardine oil had been distilled, yielding two fractions boiling below and above 215°/10 mm. respectively. The distillation had been continued up to 225°/3 mm., leaving ca. 4 kg. of residue. For the present experiments, 1.5 kg. of this residue was used as the starting material. It was first treated by means of the sodium-soap-acetone method; the sodium soaps insoluble in acetone were removed, and the highly unsaturated acids were obtained from the sodium soaps soluble in acetone in the usual way. These were converted into the methyl esters, and the latter were subjected to distillation by which all the esters boiling below 215°/2 mm. were removed. The residue was saponified, the unsaponifiable matter was removed by extraction with ether, and the fatty acids liberated from the soap solution on acidification were treated with decolorizing carbon in petroleum ether. After these treatments there were obtained 145 g. of fatty acids having neutralisation value 155.8 and

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(2) This Bulletin, **10** (1935), 232.

(3) This Bulletin, **10** (1935), 296.

(4) This Bulletin, **10** (1935), 441.

iodine value 319.1.(5) In order to effect a further separation of these fatty acids into individual components, they were dissolved in acetone and partially neutralised with sodium hydroxide solution in a similar manner as described in the previous experiment.(6) The precipitate of insoluble sodium soaps was filtered, and the fatty acids were recovered from the filtrate. These were again subjected to a fractional precipitation of sodium soaps in acetone, and the less highly unsaturated portion was removed as sodium soaps insoluble in acetone. After repeating these separative operations several times there were obtained finally 25 g. of fatty acids having iodine value 397.8 from the filtrate. These were brominated in ethereal solution, and the ether insoluble bromide was separated. On debrominating the ether insoluble bromide with zinc powder and glacial acetic acid, there were obtained 6.6 g. of fatty acids which had neutr. value 155.4 and iodine value 419.1, and were considered to consist mainly of nisinic acid. They were united with the sample (7 g.) of nisinic acid (iodine value 422.5) obtained in the previous experiment(7) and converted into the methyl esters, and the latter were subjected to distillation by which a fraction boiling at 198–204°/0.4 mm. was taken as methyl nisinate $C_{25}H_{38}O_2$. It had the following constants: d_4^{15} 0.9342, d_4^{20} 0.9307, n_D^{15} 1.5064, n_D^{20} 1.5044, saponif. value 151.1 (cal. 151.5), iodine value 408.5 (calc. 411.3).

Nisinic acid $C_{24}H_{36}O_2$ liberated from its methyl ester had the following constants: d_4^{15} 0.9487, d_4^{20} 0.9452, n_D^{15} 1.5143, n_D^{20} 1.5122, neutr. value 157.0 (calc. 157.5), iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 424.5 and 410.3 (calc. 427.5) respectively.

On brominating nisinic acid in ethereal solution it yielded 140% of an ether insoluble bromide which had Br-content 72.78% (calc. for $C_{24}H_{36}O_2Br_{12}$: 72.91%) and turned black at about 240° without melting. The hydrogenation product of nisinic acid crystallised from alcohol in lustrous laminae which showed neutr. value 152.4 (calc. for $C_{24}H_{48}O_2$: 152.3) and m.p. 82.5–83°. The melting point was not lowered when the substance was admixed with various proportions of a specimen of *n*-tetracosanic acid $C_{24}H_{48}O_2$ which was prepared from behenic acid by malonic ester synthesis and melted at 84–84.5°.

2. **Ozonolysis of Amyl Nisinate.** Nisinic acid obtained above was heated with an equal amount of amyl alcohol containing 2.5% of hydrogen chloride on the water-bath under a reflux condenser for one hour. The resulting solution containing amyl nisinate was taken up with ether, and the ethereal solution was washed with sodium carbonate solution in order to remove hydrogen chloride and the unchanged nisinic acid, and then washed thoroughly with water. On distilling off ether and amyl alcohol from the ethereal solution, there remained amyl nisinate. Nine grams of the amyl nisinate was dissolved in 90 c.c. of chloroform, and a current of ozonised oxygen was passed into the solution under cooling with ice-salt until the solution became saturated with ozone. On removal of chloroform under diminished pressure there was obtained 16.2 g. (or 180%) of ozonide as an orange yellow syrup. The ozonide was, however, not thoroughly freed from chloroform, since there was a danger of frothing towards the end of the distillation of chloroform. The theoretical yields of ozonides obtainable from amyl nisinate are 167.5% for normal ozonide $C_{29}H_{46}O_{20}$ and 171.3% for ozonide

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(5) Unless otherwise stated, the iodine values were determined by the Wijs method.

(6), (7) This Bulletin, **10** (1935), 543.

peroxide $C_{29}H_{46}O_{21}$. Water (90 c.c.) was added to the ozonide, and the liquid was heated on the water-bath for 30 minutes. The flask containing the ozonide was attached to other two flasks (**a** and **b**) in succession by means of a delivery tube; the first (**a**) was filled with ice-cold water, and the second (**b**) with barium hydroxide solution. During heating a current of hydrogen was passed through the flask containing the ozonide, and the volatile products (**A**) carried over with hydrogen were caught by absorption in the above two flasks. The products of ozonolysis remaining in the initial flask dissolved partly in water, and the remainder separated as oily substances **C**) under aqueous solution (**B**). Each portion (**A, B** and **C**) was examined in the following manner.

(i) **Volatile products (A).** The aqueous solution in the flask (**a**) produced a pink colouration with Schiff's reagent, and a deep blue colouration with sodium nitroprusside and diethylamine, indicating the presence of acetaldehyde. When the phenylhydrazone prepared from this solution was heated with zinc chloride at 180°, a smell of scatol was recognised, which indicated the presence of propyl aldehyde. A partial separation of two aldehydes was attempted by warming a portion of the solution in a flask ($\mathbf{a}_1$) at 45°, and passing a current of carbon dioxide into the flask so as to drive off the volatile substances into other flask ($\mathbf{a}_2$) which was connected with the initial flask by means of a delivery tube. The flask ($\mathbf{a}_2$) was filled with ice-cold water, which served to absorb the volatile substances carried over with carbon dioxide. The *p*-nitrophenylhydrazone prepared from the solution in the flask ($\mathbf{a}_1$) melted at 120–121° after recrystallisation from 50% alcohol, and was considered to consist largely of *p*-nitrophenylhydrazone of propyl aldehyde (Found: N, 21.84. Calc. for $C_9H_{11}O_2N_3$: N, 21.76%). The solution in the flask ($\mathbf{a}_2$) yielded *p*-nitrophenylhydrazone, which melted at 115–116° after recrystallisation from 50% alcohol and seemed to be a mixture of *p*-nitrophenylhydrazones of two aldehydes (Found: N, 22.95. Calc. for $C_8H_9O_2N_3$: N, 23.46. Calc. for $C_9H_{11}O_2N_3$: N, 21.76%).

The aqueous solution in the flask (**a**) showed also an acid reaction, and the presence of acetic acid was inferred from a red colouration developed on addition of ferric chloride to the neutralised solution.

The barium hydroxide solution in the flask (**b**) was found to contain a precipitate of barium carbonate, indicating the formation of carbon dioxide by the ozonolysis. Calculating from the quantity of the precipitate of barium carbonate, the yield of carbon dioxide formed by the ozonolysis was found to be 23.5% of amyl nisinate used for the ozonolysis. This is higher than the maximum yield 20.64% of carbon dioxide obtainable on assumption that amyl nisinate contains two of the group $=CH\cdot CH_2\cdot CH=$, and consequently amyl nisinate must have more than two of the group $=CH\cdot CH_2\cdot CH=$.

(ii) *Aqueous solution* (**B**). The products of ozonolysis dissolved in the aqueous solution (**B**) were extracted by shaking the solution with 900 c.c. of ether, and on removal of ether from the ethereal solution there remained 4.1 g. of an orange yellow liquid. This was washed three times with petroleum ether using 50 c.c. each time, and the petroleum ether solution and the insoluble portion were separated. The petroleum ether solution was heated on the water-bath to drive off petroleum ether, and the residue was then heated in the oil-bath yielding 0.7 g. of colourless distillate before the temperature of the bath reached 180°, when the distillation was discontinued. The distillate showed an acid reaction. The silver salts prepared from it were fractionally precipitated, yielding two crops which were found to be mixtures of silver acetate and silver propionate by analyses (Found for 1st crop: Ag, 61.64. Found for 2nd crop: Ag, 63.59. Calc. for $C_2H_3O_2Ag$: Ag, 64.64. Calc. for $C_3H_5O_2Ag$: Ag, 59.63%).

The portion insoluble in petroleum ether (2.5 g.) was an orange yellow liquid. Oxidation with an alkaline solution of potassium permanganate followed by acidification gave a product consisting mostly of crystalline solid. It crystallised from ethyl acetate in needles having neutr. value 947.5 (calc. for succinic acid $C_4H_6O_4$: 950.6) and m.p. 181–182°. The melting point was not lowered when the substance was admixed with a pure specimen of succinic acid (m.p. 182–183°).

(iii) *Oily substances* (**C**). Yield 3.1 g. These were oxidised with alkaline permanganate. The product obtained after acidification had neutr. value 303.1 and saponif. value 590.5, and seemed to consist mainly of amyl hydrogen succinate $C_9H_{16}O_4$ (neutr. value 298.3, saponif. value 596.5). The free acid liberated from the acid ester yielded succinic acid after recrystallisation from ethyl acetate; neutr. value 948.1 and m.p. 181–182°.

Summary.

Nisinic acid $C_{24}H_{36}O_2$ has been separated from sardine oil, and its amyl ester subjected to ozonolysis· Among the products of ozonolysis were found: propyl aldehyde, propionic acid, acetaldehyde, acetic acid, carbon dioxide, succinic acid and amyl hydrogen succinate. Of these compounds propyl aldehyde and propionic acid are derived from the group $CH_3{\cdot}CH_2{\cdot}CH{=}$, whilst acetaldehyde and acetic acid together with carbon dioxide are attributable to the secondary decomposition of malonic acid and the corresponding aldehyde which are derived from the group $=CH{\cdot}CH_2{\cdot}CH{=}$. The yield of carbon dioxide indicated the presence of more than two of the group $=CH{\cdot}CH_2{\cdot}CH{=}$. Succinic acid and amyl hydrogen succinate are derived from the groups $=CH{\cdot}(CH_2)_2{\cdot}CH{=}$ and $=CH{\cdot}(CH_2)_2{\cdot}COOC_5H_{11}$ respectively. Accordingly, nisinic acid was found to be composed of the following groups: $CH_3{\cdot}CH_2{\cdot}CH{=}$, $=CH{\cdot}CH_2{\cdot}CH{=}$ (three), $=CH{\cdot}(CH_2)_2{\cdot}CH{=}$ (two), and $=CH{\cdot}(CH_2)_2{\cdot}COOH$. The respective positions of the groups $=CH{\cdot}CH_2{\cdot}CH{=}$ and $=CH{\cdot}(CH_2)_2{\cdot}CH{=}$ were not determined in these experiments. If, however, it is assumed that three of the ethylenic linkings in nisinic acid lie at 4:5-, 8:9- and 12:13-positions as is the case with previously studied moroctic, eicosatetraenoic and clupanodonic acids, the constitution of nisinic acid is expressed by the following formula:

$$CH_3{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH{=}$$
$$CH{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}COOH.$$

Tokyo Imperial Industrial Research Laboratory,
Shibuya-ku, Tokyo.