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Precursors of methyl ketones in butter

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SUMMARY

Butterfat, when treated with Girard-T reagent, yields a series of six pyrazolones, corresponding to six even-numbered β-ketoacids (C_6 up to and including C_{16}). This is in agreement with the formation of six odd-numbered methyl ketones (C_5 up to and including C_{15}) on subjecting butterfat to steam-deodorization. The presence in butterfat of nonvolatile β-ketoacid esters has been proved. If they are glycerides, their amount is estimated at 0.03%.

In several recent publications, it is reported that when milkfat is heated in the presence of water, a homologous series of methyl ketones is produced (1, 2, 3). Patton and Tharp (3) showed that the unsaponifiable lipid of milk fat contained the same series of ketones as those found by hydrolysis at elevated temperature.

For these reasons, it is probable that milkfat contains bound β-ketoacids, as has also been suggested by Wong et al. (1). These compounds could arise biologically as intermediates of the fatty acid synthesis in the mammary gland. It seems less probable that the β-ketoacids are a primary autoxidation product.

Patton and Tharp (3) presented additional evidence for the occurrence of keto groups in milkfat, although not necessarily on the β-carbon atom of the fatty acid, with the observation of a distinct peak in the U.V. absorption spectrum at 364 mμ, following treatment of the fat with 2,4-dinitrophenylhydrazine.

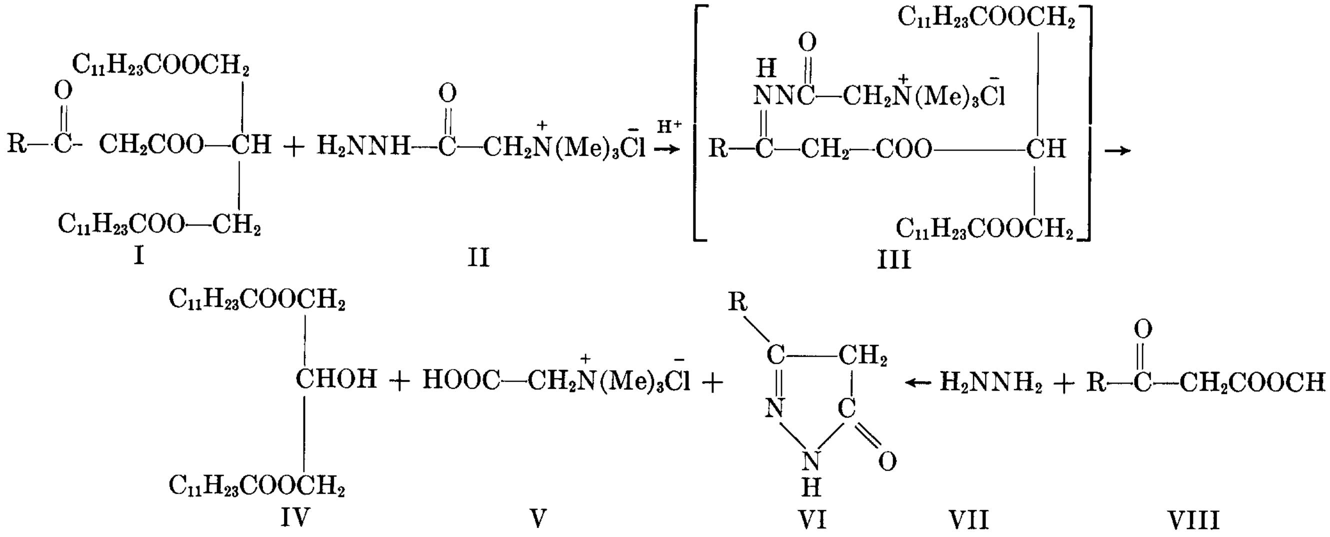
If bound β-ketoacids are the precursors of the methyl ketones found in butter, no ketones will be formed on heating butterfat in the absence of water. Vacuum degassing of dry butterfat in the apparatus of de Bruyn and Schogt (4) for 3 hr at 120° yielded only traces of ketones in the distillate, whereas heating in the presence of water yielded considerable amounts of ketones. Analogous results were obtained in a model experiment with a solution of 1,3-didodecanoyl-2-β-ketododecanoylglycerol in silicone oil.

Although these facts support the theory that β-ketoacid glycerides may be present in butter, we have tried to furnish more direct evidence through use of the Girard reagents. These reagents (5) form adducts with carbonyl compounds, which, if not always water-soluble, at least are very polar and possibly permit separation of the adducts from the bulk of the normal triglycerides.

As could be predicted (6–8), it was first established that when the Girard-T reagent (II) reacted with a typical β-ketoacid glyceride (I, $R = C_9H_{19}$), the hypothetical adduct (III) decomposed and yielded a pyrazolone (VI, $R = C_9H_{19}$) and the diglyceride dilaurin (IV). In view of the fact that the reaction product of a β-ketoacid ester with Girard-T was identical with the product produced by reaction of this β-ketoester with hydrazine, we asume that, in the reaction with Girard-T, trimethylaminoacetic acid chloride (V) is split off.

The possibility of forming pyrazolones by the reaction between butterfat and Girard-T reagent was investigated in model systems in which steam-deodorized butterfat (no longer containing precursors of ketones) served as a solvent for synthetic β-ketoacid glycerides. Solutions of a series of 3 synthetic β-ketoacid glycerides were treated with Girard-T reagent, using acetic acid as a catalyst. The pyrazolones formed were purified on an ion-exchange resin column. After washing with methanol, the low molecular weight pyrazolones could be eluted with 1 N NaOH, the long-chain ones with 0.5 N alcoholic NaOH. The yields could be calculated from absorption measurements in the ultraviolet at 242 mμ and appeared to be approximately 60%. A blank run showed that there was no pyrazolone formation in the steam-deodorized butterfat.

After reacting Girard-T reagent with butterfat, we were able to isolate a homologous series of pyrazolones;



these were separated by paper chromatography and corresponded to the ketones found after steam-deodorization of butterfat.

EXPERIMENTAL METHODS AND RESULTS

Synthesis of Model Substances. *β-Ketoacid glycerides* were prepared by heating 45 mmoles of methyl-β-ketoalkanoate, prepared according to Ställberg-Stenhagen (9) with 30 mmoles of 1,3-didodecanoylglycerol in a weak nitrogen stream at 120° according to Bader et al. (10). After cooling, the product was recrystallized once from light petroleum and three times from acetone. Infrared spectra of the reaction product showed only traces of hydroxyl absorption. To determine the 3-ketoalkanoic acid content, the products were saponified. After acidification, the mixture was refluxed and subsequently rendered alkaline. The unsaponifiable lipid was separated from the soap by extraction with purified light petroleum (prepared according to van der Ven and de Jonge (11)). The methyl ketones present in the unsaponifiable lipid were converted into the 2,4-dinitrophenylhydrazones according to Begemann and de Jong (12). After purification on a silica gel partition column with nitromethane-light petroleum according to Kramer and van Duin (13), the extinction of the dinitrophenylhydrazones was measured in chloroform at the U.V. maximum of 364 mμ ($\epsilon = 22{,}500$, the molecular extinction of the dinitrophenylhydrazones from all methyl ketones, from methyl ethyl ketone upward). The results are recorded in Table 1.

3-Alkyl-5-pyrazolones (*VI*) were prepared in the following way. 1 mmole methyl β-ketoalkanoate and 4 mmoles of Girard-T reagent in 25 ml methanol with 2.5 ml glacial acetic acid were boiled under reflux for 3 hr. Most of the methanol was removed by evaporation under a current of nitrogen. The reaction mixture was taken up in ether and washed out with water. After removal of the ether, the residue was crystallized from aqueous methanol. Table 2 shows the pyrazolones prepared. These compounds are soluble in ether and in methanol, but are only slightly soluble in light petroleum and very sparingly in water. In the infrared spectrum the following specific absorptions are noted: 1505 cm^{-1} (medium broad, —C═C—OH; i.e., enolic form), 1550 cm^{-1} (medium broad, -NH-[?], 1615 cm^{-1} (strong, cyclic -C═N bond), 2620 cm^{-1} (broad, -OH enol). The absence of a strong C═O absorption at 1700–1710 cm^{-1} supports the view that the pyrazolones are present in the enolic form. The U.V. spectrum in methanol shows two absorption maxima: at 220 and at 242 mμ ($\epsilon = 3400$). The pyrazolones are amphoteric; there is one absorption maximum at 230 in methanolic NaOH and one at 223 mμ in methanolic HCl.

For the *paper chromatography* of the pyrazolones, we used Schleicher and Schüll paper No. 2040a soaked in 1% aqueous acetic acid and subsequently dried in a hot air current. We used two solvent systems: mixture 1 consisted of cyclohexane–ethanol–water 100:85:15, and mixture 2 consisted of cyclohexane–methanol–water 100:90:10. The pyrazolones were brought on to the strip; after equilibrating for 1 hr, the ascending chromatograms were run for 2.5 hr, by which time the solvent front had moved 20–24 cm.

After drying, the paper was immersed in a 2 N sodium acetate solution, sprayed with a freshly prepared 0.1 N p-toluenediazonium chloride solution (14),

TABLE 1. β-KETOACID GLYCERIDES (I) AND METHYL KETONES OBTAINED AFTER SAPONIFICATION

Formula	R	Yield of β-Ketoacid Glycerides Based on Dilaurin	mp°	C/H Analysis Found C	C/H Analysis Found H	C/H Analysis Calculated C	C/H Analysis Calculated H	Theoretical Yield of Methyl Ketones Recovered
		%		%	%	%	%	%
$C_{35}H_{64}O_7$	C_5H_{11}	26	33–35	70.30	10.98	70.43	10.81	93
$C_{37}H_{68}O_7$	C_7H_{15}	15	50–52	71.98	11.06	71.11	10.97	92
$C_{39}H_{72}O_7$	C_9H_{19}	72	53–56	71.79	11.19	71.73	11.11	90
$C_{41}H_{76}O_7$	$C_{11}H_{23}$	74	62.5–63.5	72.64	11.43	72.30	11.25	94
$C_{43}H_{80}O_7$	$C_{13}H_{27}$	75	65.5–66.5	72.47	11.52	72.82	11.37	92

and immediately washed with water. The strip was then dried in a current of hot air. The spots were elongated and not always well separated. For the determination of the R_f values, strips of 12 mm were taken from the colored chromatograms and measured in Tennent's apparatus (15). On these strips, the absorption at 415 mμ was measured at regular short intervals. In this way, the center of the spots could be determined more accurately (cf. Table 7). Model substances were run in the same chromatogram.

Butterfat was separated from butter at 40°, dried on anhydrous $MgSO_4$, and filtered. *Milkfat* was obtained in a similar way from churned 40% sweet cream. *Degassing* experiments were carried out at 120° for 3 hr at 10^{-3} according to de Bruyn and Schogt (4). *Steam deodorization* was carried out in a laboratory deodorizer, with 25% steam at a pressure of 20 mm and at temperatures of 120° and of 180–190°. Steam distillates were trapped at −75°.

Analysis for Methyl Ketones in Butter- and Milkfats and in Model Systems. The ketones were determined by assay of the 2,4-dinitrophenylhydrazones formed in the distillates obtained after degassing or deodorization of fats. Since aldehyde dinitrophenylhydrazones are also formed from butter, these had to be separated from the ketone dinitrophenylhydrazones. In a typical experiment, the dinitrophenylhydrazones obtained from the steam distillate of 30 g butterfat were chromatographed on a 11 x 170-mm alumina column. The alumina (Imperial Chemical Industries, Ltd.) had previously been heated at 160° and 8% water added. When the column was eluted with light petroleum containing 2% diethyl ether, the ketone dinitrophenylhydrazones appeared early as a broad band.

In one experiment starting from 1 kg butterfat, the various ketone dinitrophenylhydrazones were found to correspond exactly to a series of model ketone dinitrophenylhydrazones with regard to retention volume on the silica gel partition column (13) and melting point. The mixed melting points showed no depression.

TABLE 2. 3-ALKYL-5-PYRAZOLONES (VI)

Formula	R	mp°	N Calculated	N Found
			%	%
$C_6H_{10}N_2O$	C_3H_7	195–199	22.21	22.08
$C_8H_{14}N_2O$	C_5H_{11}	190–192	18.19	18.23
$C_{10}H_{18}N_2O$	C_7H_{15}	191–192	15.38	15.56
$C_{12}H_{22}N_2O$*	C_9H_{19}	182–183	13.33	13.50
$C_{14}H_{26}N_2O$	$C_{11}H_{23}$	178–180	11.75	11.74
$C_{16}H_{30}N_2O$	$C_{13}H_{27}$	176–178	10.52	10.60

* In this compound, C and H determinations were carried out. Found, 68.46% C and 10.45% H; calculated, 68.57% C and 10.48% H.

Table 3 shows the results of model experiments with synthetic 1,3-didodecanoyl-2-β-ketododecanoylglycerol dissolved in silicone oil. In the absence of water, only traces of methylnonylketone were formed; in the presence of water, considerable amounts appeared. Table 4 gives the quantitative analysis of the individual methyl ketones from butter- and milkfats.

Reaction of a Model β-Ketoacid Glyceride with Girard-T Reagent. To a solution of 2 mmoles of I ($R = C_9H_{19}$) and 8 mmoles of Girard-T reagent in 25 ml methanol, 2.5 ml acetic acid was added and the mixture refluxed

TABLE 3. RECOVERY OF METHYL KETONES IN MODEL SYSTEMS

Substances in 30 ml Silicone Oil	Method	Yield or Recovery of Ketone
		%
0.145 g (I, $R = C_9H_{19}$)	Degassing	1.5*
Ibid.	Steam dist. 2 hr, 120°	23
Ibid.	Steam dist. 1 hr, 180°	100

* It had been demonstrated that with degassing (2 hr at 120°) of dilute solutions of methyl pentyl ketone, methyl nonyl ketone, and methyl tridecyl ketone in silicone oil, the ketones quantitatively enter the distillate.

TABLE 4. METHYL KETONES FROM BUTTER- AND MILKFATS

Fats	Method	Methyl ketones R—CO—Me (ppm) C_3H_7	C_5H_{11}	C_7H_{15}	C_9H_{19}	$C_{11}H_{23}$	$C_{13}H_{27}$
Butter	Degassing	0	0	0	0	0	0
Butter	Steam dist. 3 hr, 120°	—	2.5	2	3.5	—	—
Degassed butter Dutch I*	Steam dist. 4 hr, 190°	2.8	12.3	8.0	10.0	18.2	17.6
Milk	Degassing	—	.3	.3	—	—	—
Milk	Steam dist. 3 hr, 120°	—	2.3	1.2	1.3	—	—

* This sample had previously been freed from volatile substances by degassing in a DPi cyclic bath molecular still at a pressure of 25–40 μ and a rotor temperature of 110–120°.

for 3 hr. Subsequently, most of the methanol was evaporated under a current of nitrogen. The residue was taken up in diethyl ether and washed with water. After distilling off the ether, the mixture was extracted with light petroleum.

The dried extract weighed 915 mg, the dried residue 402 mg. The extract consisted of 1,3-didodecanoylglycerol (IV), as was proved by the hydroxyl value of 116 (theory, 123), fatty acid content of 87.6% (theory, 87.7%), and molecular weight of the fatty acid =202 (theory, 200). The light petroleum-insoluble residue was purified to mp 183° by recrystallization from aqueous methanol and from diethyl ether. The U.V. spectrum in methanol showed absorption maxima at 220 and at 242 mμ. The mixed melting point with the pyrazolone obtained from the methyl ester VI, R = C_9H_{19}; see also Table 2) was 183°.

One hundred milligrams of each of the synthetic β-ketoacid glycerides (I) was dissolved in 250 g of steam-deodorized (4 hr at 190°) butterfat. A solution of 5 g Girard-T and 2.5 ml acetic acid in 50 ml methanol was added. The reaction mixture was then refluxed in a glycerol bath at 80–85° with vigorous stirring for 8 hr. After the reaction, 750 ml light petroleum and 5 ml water were added. After thorough shaking, the lower layer was separated. The top layer was extracted with 4 portions of 55 ml methanol–water 10:1. The separated bottom layer and the four extracts were combined and, after addition of 25 ml water, were washed with light petroleum and subsequently concentrated under reduced pressure at 50–60°. The aqueous residue was extracted with diethyl ether. After drying the extract on anhydrous sodium sulfate, the ether was removed by distillation.

The residue contained the pyrazolones, which were purified in the following way. The mixture was taken up in 10 ml methanol and brought on to an 11 x 80-mm ion exchange resin column filled with Bio-Rad AG 50W-x4 in the H⁺ form, 200–400 mesh. (The column had previously been washed with 50 ml water and 50 ml methanol.) The column was eluted with 150 ml methanol, during which the pyrazolones were retained. Subsequently, low molecular weight pyrazolones could be eluted with 40 ml N NaOH (fraction 1) and the long-chain ones eluted with 40 ml 0.5 N NaOH in 50% aqueous methanol (fraction 2). The eluate was acidified with a slight excess of 2 N HCl, and then neutralized with sodium bicarbonate. The pyrazolones were extracted with ether and weighed after removal of the solvent. The purity of the pyrazolones can be checked with the U.V. spectrum. The content can be calculated from E at 242 mμ, assuming that ε = 3400. Results are shown in Table 5, from which it appears that the recovery of the added pyrazolone is not better than that of the pyrazolone formed from the corresponding β-ketoacid glycerides. The relatively low recoveries, therefore are probably not caused by incomplete reaction, but by losses during the preparative procedure.

TABLE 5. PYRAZOLONES FROM MODEL SUBSTANCES IN 250 G STEAM-DEODORIZED BUTTERFAT

Model Substances Added	Recovery of Pyrazolones Net Yield (mg)	Percentage of Theoretical
None	0	—
98.7 mg I, R = C_5H_{11}	15.7	62
100.5 mg I, R = C_9H_{19}	22.4	69
100.5 mg I, R = $C_{13}H_{27}$	18.7	50
24.7 mg VI, R = $C_{13}H_{27}$	13.8	56
24.6 mg VI, R = $C_{13}H_{27}$	12.3	50

Table 6 lists the results for various butterfats. Since the reproducibility of the R_f values is poor, a control of synthetic pyrazolones was always run on the same strip.

DISCUSSION

On treating butterfat with Girard-T reagent, a series of six pyrazolones was isolated (Table 7). These originate from the even-numbered β-ketoacids with from 6 to 16 carbon atoms and correspond to the odd-numbered C_5-C_{15} series of methyl ketones found when butterfat is steam-deodorized at 180–190° (Table 4). The formation of the pyrazolones is considered to be proof that the precursors of methyl ketones exist as nonvolatile β-ketoesters in butter.

TABLE 6. PYRAZOLONES FROM 250 g BUTTERFAT

Type of Butterfat	Pyrazolones Recovered Mg/250 g	Mean Recovery, mg/kg
Degassed Canadian	17.6 (13.3)	62
Degassed Dutch I	10.6 (14.1)	49
Degassed Dutch II	10.8	43
Fresh Dutch III	7.8 (10.7)	37
Fresh Dutch IV	12.1 (13.4)	51

TABLE 7. R_f VALUES OF PYRAZOLONES (VI) FROM BUTTER (DUTCH I) AND OF MODEL PYRAZOLONES

Solvent Mixture 1: Pyrazolones from Butter Fraction 1*	Solvent Mixture 1: Model Pyrazolones R_f	Solvent Mixture 1: Model Pyrazolones (R =)	Solvent Mixture 2: Pyrazolones from Butter Fraction 2†	Solvent Mixture 2: Model Pyrazolones R_f	Solvent Mixture 2: Model Pyrazolones (R =)
0.06	0.05	C_3H_7			
0.17	0.17	C_5H_{11}			
0.30	0.30	C_7H_{15}			
0.44	0.44	C_9H_{19}	0.26	0.25	C_9H_{19}
			0.45	0.45	$C_{11}H_{23}$
			0.60	0.60	$C_{13}H_{27}$

* Eluted from the Bio-Rad column with aqueous NaOH (see Methods).

† Eluted from the Bio-Rad column with alcoholic NaOH (see Methods).

since methyl ketones themselves do not yield pyrazolones when treated with hydrazine reagents.

Since it appears that steam-deodorization of synthetic β-ketoacid glycerides at 180° yields the ketones quantitatively (Table 3), it may be assumed that the ketones formed from Dutch butter I (Table 4) correspond to equimolar quantities of β-ketoacid glycerides in the nondeodorized fat. From these amounts of bound β-ketoacids of various chain length, 86 mg pyrazolones/kg fat should be obtained after the reaction with Girard-T reagent. If, due to difficulties in preparation, we accept a yield of 60% (Table 5), the actual amount of pyrazolones isolated should be about 52 mg/kg fat, which is in agreement with the experimental value of 49 mg/kg (Table 6).

If we assume that the precursors occur as triglycerides in which one of the acid radicals is a β-ketoacid, a total content of 0.03% in butterfat is estimated.

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