the same as previously described. The difference in volatility of the various mercaptans did not introduce any complications with this method. The weight lost with the methyl, ethyl and benzyl xanthates corresponded to loss of mercaptan and carbon oxysulfide while only carbon oxysulfide was volatile in the case of the remaining xanthates. Methyl mercaptan and carbon disulfide were lost in the case of the trithiocarbonate.

The rather high temperatures required for the decomposition of cholesteryl ethyl carbonate, acetate and chloroacetate caused slight charring when carried out at atmospheric pressure, and it was found desirable to make these runs under reduced pressure (20 mm.). The rates were the same at either pressure. The weight loss for the acetates corresponded to the acid, for the carbonate to ethanol and carbon dioxide and for the carbamate, to carbon dioxide and aniline.

The rate of decomposition of cholesteryl benzoate was determined by placing 300 mg. of the ester in a closed vial and placing it in the bath for 30 min. After the vial had been removed from the bath it was washed with benzene and ether, then opened, and its contents dissolved in 30 ml. of hot benzene. Water (50 ml.) was added and the acid was titrated with 0.05 *N* sodium hydroxide using phenolphthalein indicator.

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[CONTRIBUTION FROM THE DIVISION OF CHEMICAL RESEARCH, G. D. SEARLE AND COMPANY]

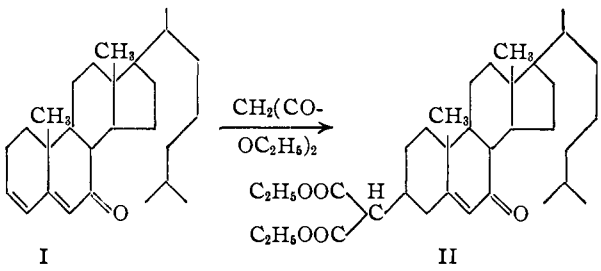
The Conjugate Addition of Ethyl Malonate to 3,5-Cholestadien-7-one

BY JACK W. RALLS

RECEIVED DECEMBER 18, 1952

Conditions for the conjugate addition of ethyl malonate to 3,5-cholestadien-7-one are described. The adduct is demonstrated to be ethyl 7-keto-3β-cholesterylmalonate. Several transformation products of the adduct have been prepared.

The stereospecific 1,6-addition of ethyl mercaptan to 3,5-cholestadien-7-one (I) has been described.[1] To examine the generality of this unusual reaction, we have studied the conjugate addition of ethyl malonate to the dienone system of I.

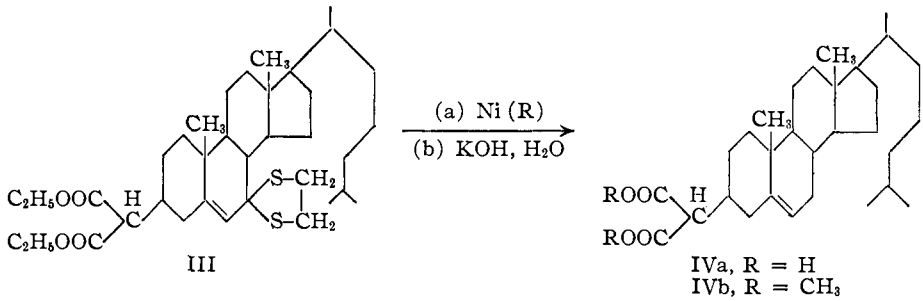


I II

There are several examples of the addition of malonic esters to α,β,γ,δ-bis-unsaturated esters.[2] The reaction of malonic esters with conjugated dienones has not been reported.

Reaction of ethyl malonate and 3,5-cholestadien-7-one (I) does not take place readily employing the milder conditions used for Michael condensations.[3] For example, a 95-hour refluxing of an equal molar mixture of ethyl malonate and I in benzene solution with piperidine as a catalyst gave no reaction. The addition proceeded at a slow rate when an ethanolic solution of the reactants containing catalytic amounts of sodium ethoxide was kept at room temperature. The most successful conditions found consisted of heating an ethanolic solution of the dienone and the active methylene compound in the presence of a molar equivalent of sodium ethoxide. In this way a 50% yield of the adduct was obtained.

The addition product (II) melted at 105–106° and had a rotation of −78°. The ultraviolet absorption spectrum (λ_{max}^{alc} 238 mμ, E_m 14,700) was excellent evidence for the structure as formulated. Confirmatory data for the assignment of structure II to the adduct and information on the stereochemical course of the reaction was obtained by converting II to the known[4,5] 3β-cholesterylmalonic acid (IVa). The ethyl 7-keto-3β-cholesterylmalonate-7-ethylenemercaptole (III) was prepared and desulfurized with Raney nickel in boiling dioxane. The intermediate ethyl 3β-cholesterylmalonate was not isolated but was saponified to 3β-cholesterylmalonic acid (IVa) melting at 199–203° and having a rotation of −30.5°. Kaiser and Svarz report,[4]



III

IVa, R = H
IVb, R = CH_3

m.p. 202–206°, $[\alpha]^{25}D$ −22.5° for this compound. The methyl ester (IVb) was also prepared and agreed with the properties described for the original preparation.

The adduct II was saponified to give 7-keto-3β-cholesterylmalonic acid (V). Thermal decarboxylation of V afforded 7-keto-3β-cholesterylacetic acid (VIa) which was characterized as the methyl ester (VIb).

Acknowledgments.—The author would like to express his appreciation to Mr. Edward A. Brown

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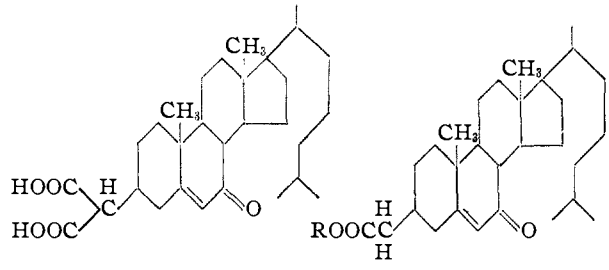

V VIa, R = H
VIb, R = CH_3

for the preparation of the ethanedithiol and to Dr. Robert T. Dillon and his staff for the analytical data. The author is indebted to Dr. Byron Riegel for his encouragement during the course of this study and for his help with the nomenclature of the compounds described.

Experimental

Ethyl 7-Keto-3β-cholesterylmalonate (II) A. Molar equivalent of sodium ethoxide.—A solution of sodium ethoxide in ethanol was prepared from 460 mg. (20 mmoles) of clean sodium and 7 ml. of dry ethanol. Then 3.1 ml. (3.21 g., 20 mmoles) of freshly distilled ethyl malonate, 7.64 g. (20 mmoles) of 3,5-cholestadiene-7-one (m.p. 112–113°) and 10 ml. of ethanol were added. The mixture was heated under reflux in a nitrogen atmosphere for 1.25 hours. Complete solution was obtained on reaching reflux temperature. The deep amber colored reaction mixture was cooled and poured into a mixture of 30 ml. of water and 2 ml. of acetic acid. The organic material was taken up in benzene. The benzene extracts were washed with water, dilute sodium bicarbonate solution and water. The benzene solution was dried over anhydrous sodium sulfate and evaporated. The orange oil which resulted weighed 10.42 g. An aliquot showed the following ultraviolet absorption characteristics: optical density at 238 mμ 0.36; optical density at 280 mμ 0.44 (concentration 0.02 mg./ml. in methanol). A 2.0-g. aliquot of the mixture was chromatographed on a column prepared from 80 g. of silica gel (Davidson Chemical Company, Grade 824). The column was packed by slurrying the silica gel in petroleum ether (30–60°). The sample was applied in 50 ml. of petroleum ether. Elution with 3:1 benzene:petroleum ether gave first 0.64 g. (43%) of essentially pure 3,5-cholestadien-7-one, after which benzene alone eluted a gummy solid, m.p. 100–103°, which on crystallization from 95% ethanol gave 1.05 g. (50%) of material melting at 105–106°. A mixed melting point with 3,5-cholestadien-7-one was 83–106°.

Anal. Calcd. for $C_{34}H_{54}O_5$: C, 75.23; H, 10.03. Found: C, 75.44; H, 9.93; $[\alpha]^{25}D$ −78° (1% in chloroform); λ_{max}^{alc} 238 mμ, E_m 14,700.

B. **Catalytic Amount of Sodium Ethoxide.**—A solution of sodium ethoxide in dry ethanol was prepared from 0.092 g. (4 mmoles) of clean sodium metal and 2 ml. of ethanol. Then 3.1 ml. (3.21 g., 20 mmoles) of ethyl malonate was added. This was followed by the addition of a solution of 7.64 g. (20 mmoles), of 3,5-cholestadien-7-one in 40 ml. of dry thiophene-free benzene. The reaction mixture was kept in the dark under nitrogen. After 112 hours, an aliquot of 9.0 ml. was taken, diluted with 60 ml. of petroleum ether (30–60°), and four small drops of acetic acid added. The solution was poured into an 80 g. of silica gel chromatography column. The chromatography was carried out exactly as described in A. The dienone fractions weighed 1.06 g. and the ketomalonate fraction weighed 0.81 g. Therefore, the extent of reaction was approximately 42%. An identical treatment of a second 9-ml. aliquot withdrawn after 256 hours gave 0.87 g. of dienone fractions and 0.93 g. of ketomalonate fraction. The extent of reaction was approximately 52% under these conditions.

C. **Piperidine as the Catalyst.**—In a nitrogen filled flask there was placed 7.64 g. (20 mmoles) of 3,5-cholestadien-7-one, 10 ml. of dry thiophene-free benzene, 3.21 g. (20 mmoles) of ethyl malonate and 0.40 ml. of freshly distilled (from sodium) piperidine. There was almost complete solution at room temperature. The mixture was heated under reflux for 95 hours in a nitrogen atmosphere. The mixture was cooled and poured into dilute hydrochloric acid. The suspension was extracted with benzene. The benzene extracts after water washing, drying, evaporation, and trituration with methanol gave 6.44 g. (84%) of material melting at 107–110°. This was identified as 3,5-cholestadien-7-one by mixed m.p.

Ethyl 7-Keto-3β-cholesterylmalonate-7-ethylenemercaptole (III).—A solution of 0.57 g. (1.05 mmoles) of ethyl 7-keto-3β-cholesterylmalonate in 5 ml. of acetic acid was treated with 0.25 g. of *p*-toluenesulfonic acid monohydrate and 0.20 ml. (2 mmoles) of ethanedithiol. After two minutes at room temperature, a crystalline solid separated. After ten minutes, the product was collected, washed with cold methanol and dried. The material weighed 0.47 g. (65%) and melted at 169–170.5°. Crystallization from acetone gave 0.42 g. melting at 169.5–179.5°.

Anal. Calcd. for $C_{36}H_{58}O_4S_2$: C, 69.86; H, 9.45; C_2H_5O, 14.56. Found: C, 69.91; H, 9.31; C_2H_5O, 14.49.

3β-Cholesterylmalonic Acid (IVa).—A suspension of freshly prepared Raney nickel (from 10 g. of the alloy) in dry purified dioxane[1] was mixed with a solution of 0.27 g. of ethyl 7-keto-3β-cholesterylmalonate-7-ethylenemercaptole in 40 ml. of dioxane. The suspension (total volume 200 ml.) was stirred and heated under reflux for six hours. After this time, an aliquot gave negative qualitative tests for sulfur (sodium fusion). The crude ethyl 3β-cholesterylmalonate was obtained as an oil after filtration and evaporation. The product weighed 0.25 g. It was suspended in 8 ml. of methanol and treated with a solution of 0.50 g. of potassium hydroxide in 3 ml. of water. More material was precipitated by the addition of the aqueous base. The mixture was heated under reflux for one hour. Complete solution resulted on reaching reflux temperature, but shortly thereafter an amorphous solid formed at the liquid level line. The suspension was cooled, acidified with dilute sulfuric acid and extracted with ether. The ethereal solution was washed with several portions of water, dried over anhydrous sodium sulfate and evaporated. The white solid which resulted weighed 0.147 g. and melted at 170–183°. Crystallization from ether:petroleum ether (30–60°) gave 39 mg. as a first crop. This material melted at 199–203° with bubbling. The rotation in 95% ethanol was −30.5°. Kaiser and Svarz report[4] m.p. 202–206° dec. with rotation of −22.5°.

Methyl 3β-Cholesterylmalonate (IVb).—A solution of 33.9 mg. of 3β-cholesterylmalonic acid in 10 ml. of ether was added to an excess of ethereal diazomethane at 5°. After ten minutes at 5°, the solution was allowed to warm to room temperature during one hour. The solution was evaporated and crystallized from ethanol. The product was collected, washed with methanol and dried at reduced pressure. The material weighed 29.8 mg. and melted at 84.5–89.5°. Another crystallization from methanol did not improve the m.p.; Kaiser and Svarz report[4] m.p. 88–89°, turbid up to 106°.

7-Keto-3β-cholesterylmalonic Acid (V).—A solution of 1.0 g. of potassium hydroxide in 5 ml. of water and 15 ml. of methanol was prepared and 0.47 g. (0.86 mmole) of ethyl 7-keto-3β-cholesterylmalonate added. The mixture was heated under reflux for one hour in a nitrogen atmosphere. Immediate solution was effected as the reflux temperature was reached. After a short reflux period a solid started separating. Considerable solid had formed by the end of the reflux period. The mixture was cooled and diluted with water and ether. The aqueous layer was pale yellow in color. The ether layer was separated and washed with water. The ether solution was dried over anhydrous sodium sulfate and evaporated. The dry weight of the neutral fraction was 0.010 g.

The combined aqueous extracts were cooled in an ice-bath and acidified with 6 *N* sulfuric acid. A precipitate separated. It was collected and washed with water. The solid was dried at reduced pressure over phosphorus pentoxide. The pale yellow solid weighed 0.40 g. and melted at about 170° with bubbling. The compound was crystallized from acetic acid–water. The purified acid melted sharply at 192° with bubbling.

Anal. Calcd. for $C_{30}H_{46}O_5$: C, 74.03; H, 9.53. Found: C, 74.02; H, 9.74; λ_{max}^{alc} 238 mμ, E_m 16,000.

7-Keto-3β-cholesterylacetic Acid (VIa).—A 50-ml. distillation flask was charged with 1.38 g. (3.40 mmoles) of 7-keto-3β-cholesterylmalonic acid and evacuated to a pres-

sure of 0.05 mm. The flask was slowly lowered into a Wood metal bath as the temperature of the bath was slowly raised from 170° to 195°. After five minutes, the temperature was 195°. The bath was held at 195–200° for 15 minutes with the bulb portion of the flask totally immersed. The solid acid melted with vigorous frothing and bubbling for the first ten minutes of heating. After 15 minutes the gas evolution had practically ceased. On cooling the material solidified. The substance was dissolved in boiling methanol, filtered, and cooled. The product was collected, dried and weighed. The material (0.49 g., 40%) melted at 155–160°. Several crystallizations from methanol raised the melting point to 167–169°.

Anal. Calcd. for $C_{29}H_{46}O_3$: C, 78.68; H, 10.48. Found: C, 78.43; H, 10.47; λ_{max}^{cal} 238 mμ, E_m 13,300; $[\alpha]_D^{25}$ − 103.5° (1% chloroform).

Methyl 7-Keto-3β-cholesterylacetate (VIb).—The filtrate from the first crop isolation of the 7-keto-3β-cholesterylacetic acid described above was diluted with methanol to a volume of 80 ml. The solution was poured into a nitrogen-filled flask and three small drops of sulfuric acid added. After standing for 16 hours at room temperature, the solution was heated under reflux for 5 hours, cooled, and poured into water. The suspension was extracted with benzene. The combined benzene extracts were washed with water and dried over anhydrous sodium sulfate. The benzene solution (120 ml.) was poured into a silica gel (50 g.) chromatography column. Elution with 9:1 benzene:ethyl acetate gave 0.64 g. of material in the only elution peak. Trituration of this material with methanol gave 0.53 g. of solid which melted at 102–104°. Crystallization from methanol gave large puffs of cotton-like material. This compound melted at 103.5–105°.

Anal. Calcd. for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59. Found: C, 78.92; H, 10.56; λ_{max}^{alc} 238 mμ, E_m 14,100; $[\alpha]^{25}D$ −87° (1% in acetone).

CHICAGO 80, ILLINOIS

[CONTRIBUTION FROM THE MEDICINAL CHEMISTRY BRANCH, CHEMICAL CORPS MEDICAL LABORATORIES]

Model Reactions of Phosphorus-Containing Enzyme Inactivators. III.[1] Interaction of Imidazole, Pyridine and Some of their Derivatives with Dialkyl Halogeno-phosphates

BY T. WAGNER-JAUREGG AND B. E. HACKLEY, JR.

RECEIVED OCTOBER 16, 1952

The hydrolysis of diisopropyl fluorophosphate and of diethyl fluorophosphate can be accelerated by imidazole, histidine, pyridine and by certain of their derivatives. A comparison of their activity is made and the possibility is discussed that the catalytic function of the investigated amines might be explained by the formation of an intermediary quaternary complex between the tertiary nitrogen and the dialkyl fluorophosphates. N-Diisopropylphosphoryl imidazole has been synthesized and its spontaneous hydrolysis studied. A hypothesis is presented that two different essential centers may be involved in the reaction of DFP with enzymes: one group which functions as a phosphorylation catalyst, perhaps of the imidazole type, and an ultimate acceptor for the phosphoryl group.

In the inactivation of chymotrypsin by diisopropyl fluorophosphate (DFP), a diisopropylphosphoryl–chymotrypsin is formed by replacement of one reactive hydrogen atom of the enzyme by the $PO(OR)_2$ group[2]

$$\text{chymotrypsin} + FPO(OR)_2 = \text{chymotrypsin} - PO(OR)_2 + HF$$

After treatment of the phosphorylated chymotrypsin with 2 *N* hydrochloric acid at 100° for several hours, phosphorus of the bound DFP has been isolated as serine phosphate.[3] It cannot be considered definitely established that the phosphoryl group reacts directly with the OH group of serine during the inactivation process; the isolated serine phosphate could be an artefact, produced during the process of hydrolysis. The study of model reactions with halogeno-phosphates might be helpful for a better understanding of the reaction mechanism.

We have never been able to isolate phosphorylated products from serine or its methyl ester after treatment with DFP. In chloroform solution, serine methyl ester is phosphorylated on the nitrogen atom by diisopropyl chlorophosphate (DClP).[4a,b] The possibility remains that there may be specific catalytic centers present in proteins which induce the phosphorylation of groups which, in an isolated form, are not reactive with DFP.

In order to study this problem, the manometric method of measuring the amount of CO_2 produced by interaction of DFP and a substrate in a bicarbonate–CO_2 buffer was used. This technique had been applied previously in an investigation of the reactivity of phenolic substances with DFP.[5] A phosphorylation reaction is characterized by the evolution of one mole of CO_2 per mole of disappearing dialkyl fluorophosphate, according to equation A. Two moles of CO_2 is expected in the case of hydrolysis of DFP (equation B)

$$(RO)_2POF + R'OH + NaHCO_3 = (RO)_2POOR' + NaF + H_2O + CO_2 \quad (A)$$

$$(RO)_2POF + 2NaHCO_3 = (RO)_2POONa + NaF + H_2O + 2CO_2 \quad (B)$$

$$2(RO)_2POF + 2NaHCO_3 = (RO)_2OPOPO(OR)_2 + 2NaF + H_2O + 2CO_2 \quad (C)$$

The formation of tetraalkyl pyrophosphate would be characterized by the liberation of one mole of CO_2 per one mole of dialkyl fluorophosphate (equation C). This possibility certainly can be excluded when diethyl fluorophosphate is used since the

(1) Some of the results of this paper were presented at the Meeting of the American Society of Biological Chemists, at New York, N. Y., April, 1952; abstract, *Federation Proc.*, **11**, 224 (1952). For two preceding publications see footnotes 4a and 5.

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