SYNTHESIS OF ETHYL [$^{14}CH_3$]METHYLMALONYL THIOGLYCOLATE AS A POSSIBLE SUBSTRATE ANALOGUE OF [$^{14}CH_3$]METHYLMALONYL COENZYME-A

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SUMMARY

Ethyl methylmalonyl thioglycolate is a potential substrate analogue of methylmalonyl-coenzyme-A (methylmalonyl-CoA) in the investigation of propionic acid metabolism. To prove this hypothesis, the tracer ethyl [$^{14}CH_3$]methylmalonyl thioglycolate was synthesized via methyl-Meldrum's acid to carry out the biochemical examinations. The method described can also be used to synthesize [$^{14}CH_3$]methylmalonyl-CoA by transesterification of active labelled methylmalonyl thiophenyl ester. This latter intermediate is chemically stable when stored at room temperature, and the unstable [$^{14}CH_3$]methylmalonyl-CoA can be prepared in one step just preceeding the biochemical experiments.

Key words: ethyl [$^{14}CH_3$]methylmalonyl thioglycolate, [$^{14}CH_3$]methyl-Meldrum's acid, [$^{14}CH_3$]methylmalonyl thiophenolate, [$^{14}CH_3$]methylmalonyl-CoA.

INTRODUCTION

Investigations have been accomplished [1,2,3] to clarify the mechanism of decomposition of propionyl-CoA via the methylmalonate reaction pathway shown in Fig. 1 [4]. The irregular function of the given enzymes can cause serious illnesses in humans such as aciduria. In addition, the fermentation industry also requires the exact knowledge of this metabolic pathway, for example in the case of erythromycin production by Streptomyces erythreus. Namely, it was found [5] that the methylmalonyl-CoA intermediate is formed in the citrate cycle through succinyl-CoA. This and similar investigations are very often based on tracer techniques.

The compound generally used in these investigations is the [$^{14}CH_3$]-labelled methylmalonyl-CoA which has to be synthesized for the biochemical experiments. The non-labelled compound is commercially available and it undergoes approximately 1.5 %

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decomposition per week at -12 °C storage temperature. Under the conditions of the usual biochemical experiments a decomposition of 4 % per hour was observed.

These values are not critical, nevertheless if compared with the high price of CoA it seems reasonable to find a compound which behaves as a substrate analogue in preliminary measurements of the enzyme activity. For this purpose the [$^{14}CH_3$]-labelled ethyl methylmalonyl thioglycolate was synthesized. In the procedure described below, several active esters, among them [$^{14}CH_3$]methylmalonyl thiophenolate, were prepared. The latter compound can be stored at room temperature for long periods of time, and can be easily transesterified with CoA in a single step prior to the biochemical experiments.

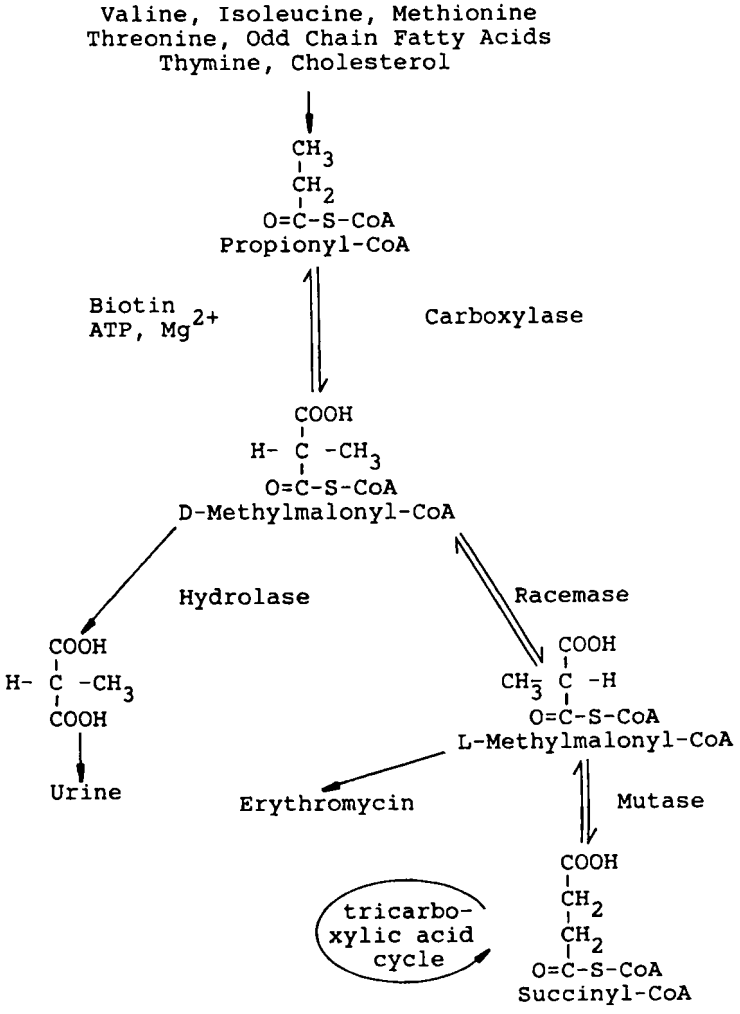


Fig. 1. Biochemical pathway of methylmalonyl-CoA

SYNTHESIS OF ETHYL [$^{14}CH_3$]METHYLMALONYL THIOGLYCOLATE

Racemic ethyl [$^{14}CH_3$]methylmalonyl thioglycolate of ≥ 8 MBq/mM specific activity was required for a series of biochemical experiments. All the previously known methods use methylmalonic acid to form the intermediate compounds during the syntheses. It was prepared by methylation of diethyl malonate followed by hydrolysis. Labelling was carried out during methylation with ^{14}C labelled CH_3Br ($^*CH_3Br$).

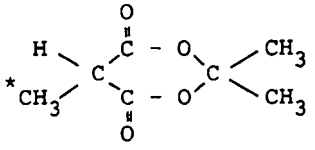
1.a. $H-CH(COOC_2H_5)_2 \xrightarrow[Na]{\text{ethanol}} H-C(COOC_2H_5)_2Na \xrightarrow[0\ ^oC]{^*CH_3Br} H-C(COOC_2H_5)_2{}^*CH_3$

b. $2\ {}^*CH_3OH + 2\ NaBr + H_2SO_4 \xrightarrow[CH_3OH]{75\ ^oC} 2\ {}^*CH_3Br + Na_2SO_4 + 2\ H_2O$

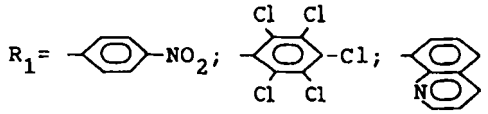
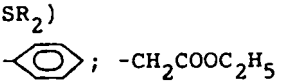
2. $H-C(COOC_2H_5)_2{}^*CH_3 \xrightarrow[KOH]{\text{ethanol}} H-C(COOK)_2{}^*CH_3 \xrightarrow{HCl} H-C(COOH)_2{}^*CH_3$

The previously reported methods for the synthesis of ethyl methylmalonyl thioglycolate (employing mixed anhydride [3], dicyclohexylcarbodiimide [6] or acyl chloride [7,8]) resulted in low yields (see Table 1). It was therefore necessary to find a new procedure. with improved yield.

Junek and coworkers described [9] the possibility for the synthesis of monoesters of malonic acid via Meldrum's acid. Several alcohols and thioalcohols were utilized to synthesize monoesters by this method via methyl-Meldrum's acid in the following way:

3. $H-C(COOH)_2{}^*CH_3 \xrightarrow[< 20\ ^oC]{\text{acetone, } (CH_3CO)_2O,\ H_2SO_4}$ 

4. Methyl-Meldrum's acid $\xrightarrow[120\ ^oC]{R_1OH,(R_2SH)}$ $H-C(COOH)(O{=}C{-}OR_1(SR_2)){}^*CH_3$ + acetone

R_1=  ; R_2=  ; $-CH_2COOC_2H_5$

The yields of monoesters obtained from methylmalonic acid by different methods are compared in Table 1.

Thioalcohols / Alcohols / Methods	p-NO_2-phenol	Pentachlorphenol	8-hydroxyquinoline	Thiophenol	Ethyl thioglycolate
mixed anhydryde	-	-	-	2	2
dicyclohexylcarbodiimide	5	5	2	3	3
acyl chloride	2	3	-	1	2
methyl-Meldrum's acid	22	20	6	24	25

Table 1. The yields (in %) of different monoesters and monothioesters synthesized from methylmalonic acid by different methods.

As indicated in Table 1, the method via methyl-Meldrum's acid gave significantly better yield for all cases. Most of the synthetic steps were known but some of them had to be modified, and purification methods had to be worked out in order to obtain pure intermediates.

1. Methylation of diethyl malonate was carried out as described by Weiner [10] with the following modifications:

i. it was found that filtration of NaBr after the methylation (Reaction 1.a.) was not necessary and had no effect on the yield and the quality of the product.

ii. because of the low b.p. of CH_3Br (3,5 ^{o}C) the reaction mixture was cooled to 0 ^{o}C in order to condense the CH_3Br gas. In this way the excess of CH_3Br was considerably reduced (from 8x to 2x) and proportionally higher specific activity was achieved in the final product. However, because of the lower rate of reaction at 0 ^{o}C, methylation required 4 hours. The yield of conversion was 85 % on average, and there was 8-10 % unmethylated and no dimethylated product in the reaction mixture. This was the reason why we chose CH_3Br for methylation instead of CH_3I, excess CH_3I giving rise to dimethylated by-product [11].

2. The hydrolysis of [$^{14}CH_3$]methylmalonic ester was carried out in an alcoholic mixture of KOH [12], followed by recrystallization from ethyl acetate/petroleum ether. Ethyl acetate dissolves only a

small quantity of malonic acid, therefore the recrystallized product is essentially pure [$^{14}CH_3$]methylmalonic acid.

3. To synthesize the [$^{14}CH_3$]methyl-Meldrum's acid the method of Meldrum was used as modified by Davidson [13].

4. The preparation of the monoesters of methylmalonic acid [9] was carried out with the following modifications:

i. the optimum temperature for production of monoesters and monothioesters was 120 °C.

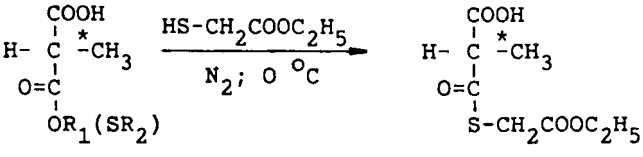
ii. the necessary reaction time was found to be 70 minutes (60 minutes at atmospheric pressure and 10 minutes in vacuum).

iii. since a previous report [9] did not describe a purification step for the final product, a method was developed for the separation of monoesters from the reaction mixture based on the solubility of monoesters in alkaline solution (see Experimental).

The method described above was suitable for synthesizing all the monoesters and monothioesters shown in Table 1, among them the ethyl methylmalonyl thioglycolate. Methylmalonyl-CoA itself can not be prepared directly this way because of the sensitivity of the CoA to heat.

Vagelos [14] described a procedure for the transesterification of methylmalonyl thiophenyl ester to methylmalonyl-CoA based on the enhanced reactivity of the active esters. The modified reaction is as follows:

5.



The reaction temperature was kept lower than in [14] and nitrogen was bubbled through the reaction mixture for 3 hours. For separation and purification of ethyl methylamalonyl thioglycolate, the same method was used as in step 4.

Since ethyl methylmalonyl thioglycolate was intended not only as a substrate analogue in preliminary biological experiments, but also for modeling the labelled methylmalonyl-CoA synthesis, the first four esters, synthesized in steps 1 - 4 (Table 1), were

transesterified according to reaction 5. The aim was to find a chemically stable active ester which gives an acceptable yield. The results are given in Table 2.

Active esters	p-NO_2-phenyl ester	8-hydroxy-quinoline ester	pentachloro-phenyl ester	thiophenyl ester
yields (%)	15	4	20	58

Table 2. Yields of transesterification of several active esters to ethyl methylmalonyl thioglycolate

As indicated in Table 2, the best yield was obtained by using methylmalonyl thiophenyl ester for transesterification. Since this ester afforded the best yield in steps 3 and 4 (Table 1), it was chosen for the synthesis of methylmalonyl-CoA. Moreover the thiophenyl ester is very stable with no appreciable decomposition observed within one year.

The overall yield for the ethyl methylmalonyl thioglycolate was 10% after transesterification. In comparison if it was prepared not by transesterification but directly by steps 1-4 the overall yield was 17%.

EXPERIMENTAL

1. Methylation of diethyl malonate

Sodium (1.15 g, 50 mg atom) was dissolved in 28 ml absolute ethanol in a flask equipped with a magnetic stirrer, reflux condenser and calcium chloride tube. Diethyl malonate (8 g, 50 mM) was added to the solution then ^{14}C-labelled CH_3Br (9.5 g, 50 mM) was bubbled through it and stirred at 0 ^{o}C. The addition of methyl bromide lasted about 4 hours after which the reaction mixture was refluxed for an additional hour. The major part of the alcohol was then evaporated at atmospheric pressure. Sodium bromide was dissolved in 0.5 M HCl after which two phases were obtained. The upper layer contained the methylated product, and was used for hydrolysis without purification. The yield was 85%.

The [^{14}C]CH_3Br was produced as follows:

Sulphuric acid (10.3 g, 95%) was mixed at 0 ^{o}C with methanol (6.4 g, 200 mM), containing 200 MBq [^{14}C]methanol. Sodium bromide

(19 g) was suspended with a small portion of this mixture in a 40 ml flask, equipped with a dropping funnel and a gas inlet tube. The release of the labelled CH_3Br gas was started by heating the flask on a water bath up to 75 °C. The methanol-acid mixture was then added in small portions from the funnel to maintain slow introduction of the CH_3Br gas into the reaction vessel. When the gas evaluation decreased the temperature was slowly raised until no more methyl bromide was generated.

2. Hydrolysis of the methylmalonic ester

The ester (6.96 g, 40 mM) was dissolved in 20 ml ethanol - 10.0 ml water mixture containing KOH (7.84 g) in a flask equipped with a reflux condenser. The mixture was boiled with stirring on a water bath for 4 hours, then the alcohol was evaporated. The residual precipitate was dissolved in water and the mixture was acidified with HCl (pH=1), extracted with ether (4x10 ml), the ether layer dried over $CaCl_2$, filtered and evaporated. The residue was recrystallized twice from ethyl acetate/petroleum ether. The yield was 80%.

3. Preparation of [$^{14}CH_3$]methyl-Meldrum's acid

Dry [$^{14}CH_3$]methylmalonic acid (3.54 g, 30 mM) was suspended in acetic anhydryde (1.584 g, 36 mM) and 0.15 ml of conc. sulphuric acid was added with stirring and cooling. After the [$^{14}CH_3$]methylmalonic acid was dissolved, acetone (1.914 g, 33 mM) was added to this solution while the temperature was kept below 20 °C. The reaction mixture was kept in the refrigerator for one day and the obtained methyl-Meldrum's acid crystals were filtered by suction and washed three times with ice-water sufficient to cover the cake. The yield of this step was 80%.

4. Preparation of [$^{14}CH_3$]methylmalonyl thiophenolate

This procedure was employed for the synthesis of another active esters and for the direct preparation of ethyl methylmalonyl thioglycolate on the same scale.

Dry [$^{14}CH_3$]methyl-Meldrum's acid (2.37 g, 15 mM) and thiophenol (1.65 g, 15 mM) were mixed in a rotary evaporator at 120 °C for 70 minutes. In the last 10 minutes the reaction vessel was evacuated.

The residue was dissolved in saturated $NaHCO_3$ solution and extracted with 3x10 ml of ether in order to remove the diester by-products. The aqueous phase was acidified with HCl (pH=1) and then extracted with ether (3x15 ml). The ether phase was dried over $CaCl_2$, filtered and evaporated. The product was recrystallized from ethyl acetate/petroleum ether. The yield was 30 %.

The quality control of the intermediates was carried out in all stages by HPLC analysis, elemental analysis, ^{1}H-NMR spectral measurements and, in case of available literature data, melting point control.

Fig. 2 shows the ^{1}H-NMR spectrum of the methylmalonyl thiophenyl monoester :

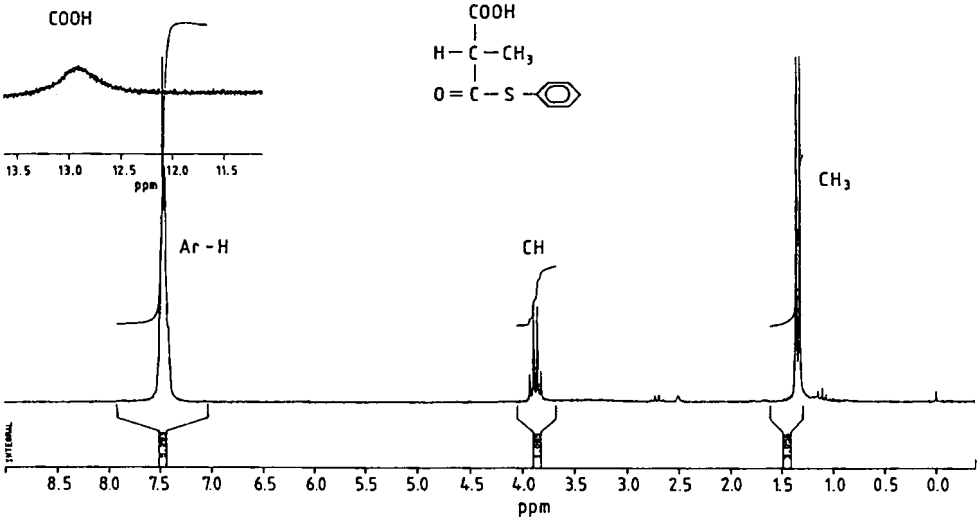


Fig. 2. The ^{1}H-NMR spectrum of the methylmalonyl thiophenyl monoester.

5. Transesterification

Ethyl thioglycolate (0.372 g, 3.1 mM) was dissolved in 10 ml of 0.1 M $NaHCO_3$ buffer at pH=8.5 and added to [$^{14}CH_3$]methylmalonyl

thiophenyl ester (0.63 g, 3 mM) dissolved in 0.1 M $NaHCO_3$ solution (10 ml). Both mixtures were previously cooled to 0 ^{o}C while nitrogen gas was bubbled through them. After 3 hours the solution was made alkaline (pH=10) and extracted with ether (3x10 ml). The aqueous phase was acidified with conc. HCl (pH=1) and extracted with ether (3x15 ml). The ether phase was dried over $CaCl_2$, filtered and evaporated.

The product was purified by recrystallization from ethyl acetate/ petroleum ether. The ^{1}H-NMR spectrum of the trans-esterified product is shown in Fig. 3. The direct synthesis of ethyl methylmalonyl thioglycolate in step 4 gives the same compound which was proved by quality control.

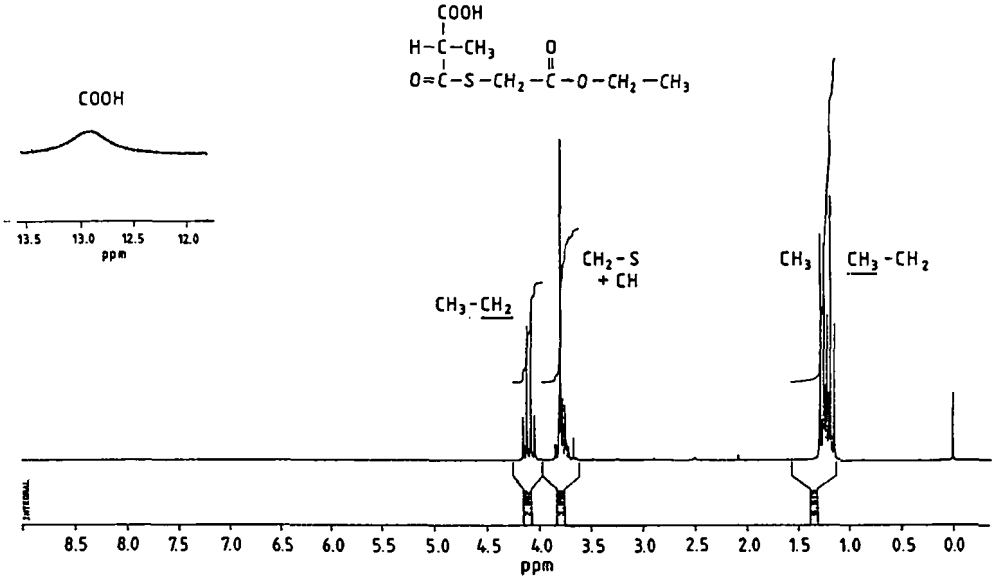


Fig.3. The ^{1}H-NMR spectrum of the ethyl methylmalonyl thioglycolate.

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REFERENCES

1. M. Flavin, S. Ochoa - J. Biol. Chem. 229:965(1957)
2. M. Flavin, H. Castro-Mendoza, S. Ochoa - J. Biol. Chem. 229:981(1957)
3. W.S. Beck, M. Flavin, S. Ochoa - J. Biol. Chem. 229:997(1957)
4. S.P. Stabler, P.D. Marcele, R.H. Allen - Arch. Biochem.Biophys. 241:252(1985)
5. A.A. Hunaiti, P.E. Kolattukudy - Antimicrobial Agents and Chemotherapy 25:173(1984)
6. H.G. Khorana, W.E. Razzel, P.T. Gilham, G.M. Tener, E.M. Pol - J. Am. Chem. Soc. 79:1002(1957)
7. P. Overath, E.R. Stadtman, G.M. Kellerman, F. Lynen - Biochemische Zeitschrift 336:77(1962)
8. P. Overath, G.M. Kellermann, F. Lynen - Biochemische Zeitschrift 335:500(1962)
9. H. Junek, E. Ziegler, U. Herzog, H. Kroboth - Synthesis - 8:332(1976)
10. Organic Syntheses, Coll. Vol.2, p. 279.
11. J. Benes, M. Dobiasova, K. Veres - Collect. Czech. Chem. Commun. 29:3178(1964)
12. ORGANIKUM, Organisch-chemisches Grundpraktikum, 9.überarbeitete Auflage - VEB Deutsches Verlag der Wissenschaften, Berlin, 1970.
13. D. Davidson, S.A. Bernhard - J. Am. Chem. Soc. 70:3426(1948)
14. P. R. Vagelos - J. Biol. Chem. 235:346(1960)