NEAR-QUANTITATIVE CONVERSION OF LABELLED ACIDS TO ESTERS BY MODIFIED HASSNER ESTERIFICATION. SYNTHESIS OF LABELLED TRIGLYCERIDES.

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The modification of the reaction conditions of Hassner esterification - DCC/4-dimethylaminopyridine method - merely the gradual addition of DCC to the reaction mixture - eliminated the unwanted side reaction - N-acyl-N,N´-dicyclohexylurea formation - and thus improved the conversion of the acid to the desired ester. Glycerol tri[U-^{14}C]palmitate, glycerol tri[U-^{14}C]oleate and glycerol tri[9,10^{3}H]stearate with molar activities greater than 1800 MBq.mmol^{-1} were prepared with preparative radiochemical yields about 86-92 %.

Key words: Glycerol tri[U-^{14}C]palmitate, glycerol tri[U-^{14}C]oleate, glycerol tri[9,10-^{3}H]stearate, esterification, labelled acids, triglycerides.

INTRODUCTION

Triglycerides labelled in the fatty acid portion by the radionuclide ^{14}C find application in clinical biochemistry in the so called "breath-test". After per os administration of labelled triglyceride the rate of [^{14}C]CO_2 elimination in expired air is measured and any malfunctions of the gastro-intestinal tract

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can be detected (1,2,3,4). Usually triglycerides labelled with [1-^{14}C] fatty acids were used. However, Weinman and coworkers observed that the rate of the [^{14}C] CO_2 elimination from the organism of rats is the same after the administration of either glycerol tri[1-^{14}C] palmitate, glycerol tri[6-^{14}C] palmitate or glycerol tri[11-^{14}C] palmitate (5). From this fact they have drawn a conclusion, that after the first β-oxidation the whole palmitic acid is quickly destroyed to low molecular fragments (the same is not true for stearic acid (6)). Thus glycerol tri[U-^{14}C] palmitate can be used in the "breath test".

Chemical synthesis of triglycerides followed the progress in esterification reactions. The first syntheses (7,8) describe the formation of triglycerides by heating a glycerol - fatty acid mixture to 160-190 °C while reducing the pressure to 1.4-2 kPa (10-15 torr). Better yields were achieved using p-toluene sulfonic acid as a catalyst (9,10) under similar reaction conditions or 2-naphthalene sulfonic acid in refluxing xylene with azeotropic water removal (11). Unique is the paper describing the formation of triglycerides by the reaction of the silver salt of the fatty acid with 1,2,3-tribromopropane in refluxing xylene (12).

The more frequently used method consists in conversion of the fatty acid to its chloride using PCl_5, $SOCl_2$ or $(COCl)_2$ and reaction of fatty acylchloride with glycerol in chloroform--pyridine mixture (13,14,15), yields vary from 50 to 70 %. The synthesis of glycerol tripalmitate from free palmitic acid and glycerol by the action of trifuoroacetic anhydride (16) was described with 70 % yield.

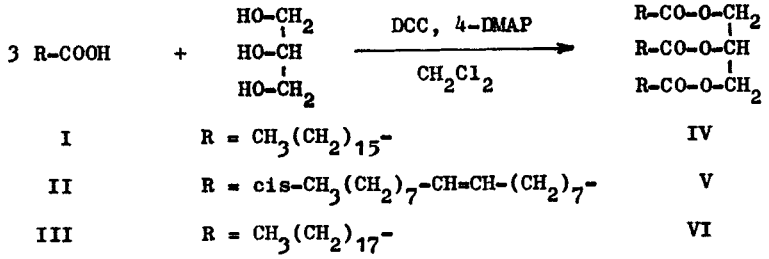
When choosing the suitable synthetic method for the synthesis of triglycerides labelled with radionuclides our aim was to synthesize not only triglycerides with molar activities of the order of 2000 MBq.mmol^{-1} but also with molar activities up to 20 times higher and thus profit from high molar activity of [U-^{14}C] fatty acids.

In the year 1978 Hassner et al. described the esterification of free organic acids with alcohols by action of dicyclohexylcarbodiimide (DCC) in the presence of 4-dialkylaminopyridine under very mild reaction conditions (17,18).

This method was successfully applied to the synthesis of fatty acid esters of cholesterol in 75 % yield based on the acid (19) and we decided to apply this method for the synthesis of labelled triglycerides on the submillimole scale.

RESULTS AND DISCUSSION

In the pilot experiment when exactly the molar ratio of reagents described in the original paper (17), i.e. 3.3 mmol of DCC, 0.3 mmol of 4-dimethylaminopyridine (4-DMAP), 1 mmol of glycerol and 3 mmoles of palmitic acid (I) were used, the yield of corresponding glycerol tripalmitate (IV) was only 50 %.

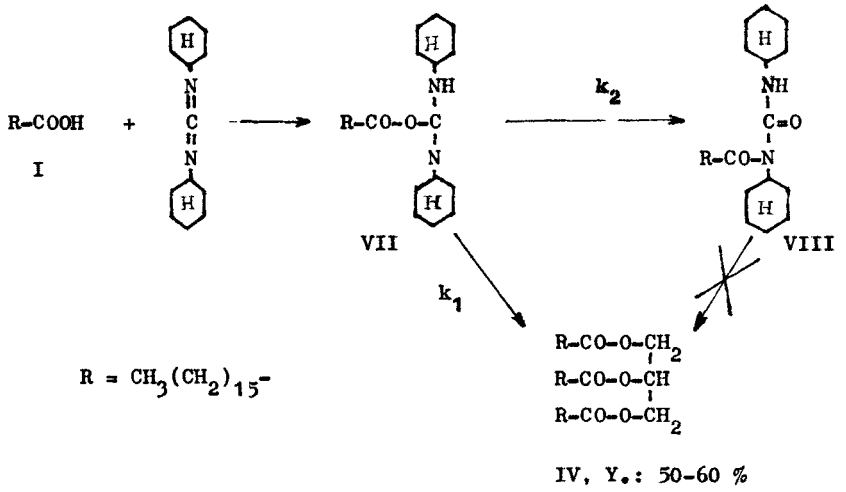


SCHEME 1

In the tracer experiment with [U-^{14}C]palmitic acid it was prooved, that the [U-^{14}C]palmitoyl is present in the isolated side product. The structure of this side product was assigned on the basis of IR, ^{1}H NMR and elemental analysis as a N,N´-dicyclohexyl-N-palmitoyl-urea (VIII).

This side reaction - the rearrangement of primarily formed O-acyl isourea VII to N-acylurea VIII - is the drawback of the

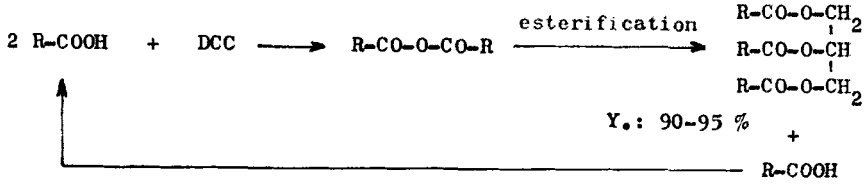
use of DCC as a condensing agent and was described by several authors (20,21,22).



SCHEME 2

As it is seen from Scheme II the yield of triglyceride depends on k_1/k_2 ratio. Thus increasing the k_1 by the addition of equimolar amount of esterification catalyst - DMAP - leads to an improvement of the triglyceride yield by 20 %.

A further 20 % increase in yield was obtained when DCC was added gradually to the reaction mixture, the O-acyl isourea (VII) reacts with excess acid very quickly to yield the fatty acid anhydride. This then acylates glycerol and the liberated acid is converted to the anhydride by a further portion of the DCC. Thus by adding successively 0.5, 0.25, 0.125 and two 0.06 mmol portions of DCC to 1 mmol of the fatty acid a better than 90 % conversion of these fatty acids to glyceroltriester was achieved (23).



SCHEME 3

The triglycerides were separated from the reaction mixture by flash-chromatography using a silica gel column. The identity of isolated non labelled glycerol tripalmitate followed from its melting point, mixed melting point and from comparison of its IR spectra with that of a standard. The prepared labelled triglycerides were characterised by their TLC behavior identical with that of respective non labelled triglyceride standards. Radiochemical purity of the prepared triglycerides checked by radio-TLC was always greater than 98 %.

EXPERIMENTAL

Melting points were determined on microstage Boetius (Rapido, Dresden). IR spectra were recorded on UR-20 (Zeiss, Jena) in CCl_4 (conc. 5 %); ^1H NMR spectra were measured in $CDCl_3$ on TESLA (Brno) apparatus at 60 MHz with TMS as an internal standard (δ scale, interaction constants in Hz). Samples for analysis were dried over phosphorus pentoxide at 13 Pa pressure. TLC was performed on SILUFOL (Kavalier, Votice) plates in n-hexane-diethylether - acetic acid (80:20:1, v/v) mixture. Lipids were detected by spraying the plate with a 5 % solution of phosphomolybdate acid in ethanol and heating as a blue-black spots on greyish-green background. Activity distribution on plates was measured using a TLC-Scanner II Berthold (Wild bad) combined with the multichannel analyzer Berthold--Silena and HP 97-S calculator. R_f´s of compounds are given in Table 1.

1,2-Dichloroethane was fractionated from P_2O_5 and stored in an Amber bottle over molecular sieves. A stock solution of DCC was made in dry benzene. Materials for flash-chromatography were from J.T.Baker (Phillipsburg).
Standard palmitic acid, oleic acid, stearic acid, glycerol tripalmitate, glycerol trioleate and glycerol tristearate were obtained from Sigma (St.Louis).

[U-^{14}C]Palmitic acid, [U-^{14}C]oleic acid with molar activities greater than 21 GBq.mmol^{-1} and [9,10-^{3}H]stearic acid were purchased from ÚVVVR Prague.

Activities were measured on a Packard 2660 apparatus using a liquid scintillator SLD-31 (dioxane based, Lachema Brno). Corrections for quenching were determined by the channels ratio method using standards made by ÚVVVR Prague.

TABLE 1

R_f Values of described lipids in n-hexane- diethylether - - acetic acid (80:20:1 v/v) mixture on SILUFOL (Kavalier-Votice)

Compound		R_f
glycerol tripalmitate	(IV)	0.72
glycerol trioleate	(V)	0.70
glycerol tristearate	(VI)	0.63
palmitic acid	(I)	0.48
oleic acid	(II)	0.26
stearic acid	(III)	0.34

Non-active glycerol tripalmitate (IV)

To the flask equipped with a magnetic stirrer and adapter for working in an inert atmosphere 256 mg (1 mmol) of palmitic acid (I), 20 µl (0.27 mmol) of glycerol, 412 mg (2 mmol) of DCC and 33 mg (0.27 mmol) of DMAP were added. The reaction flask was flushed with argon and via a silicon septum 10 ml of chloroform, freshly distilled from P_2O_5, were injected. The reaction flask was covered by an Al-foil and the mixture was stirred 48 hours at ambient temperature. The reaction mixture was washed successively by dilute hydrochloric acid, saturated $NaHCO_3$ solution and water. The organic layer was dried by $CaCl_2$, concentrated and applied to a silica gel

column. Elution by n-hexane - diethylether (80:20, v/v) gave 110 mg (50 %) of glycerol tripalmitate (IV) which was recrystallized from ether m.p. = 62.5 - 64 °C (Lit.(16) m.p.: 66 °C). IR: 1162 cm^{-1} (-C-O-), 1748 cm^{-1} (C=O).

Further elution gave 170 mg (31 %) of N,N´-dicyclohexyl--N-palmitoyl urea (VIII), m.p.: 65.5-67.5 °C, after recrystallization from ethanol-diethylether-petrolether mixture m.p.=68-69 °C.

IR: 1530 cm^{-1}, 1640 cm^{-1}, 1710 cm^{-1} (-NH-CO-N-CO-R), 3290 cm^{-1}, 3440 cm^{-1} (-NH-CO-)

^{1}H NMR: 0.87 bs (3H, $-CH_3$), 1.25 bs (46 H, $-CH_2-$), 2.33 d (2H, $-CH_2-CO-$, $J_{-CH-CH-CO-}$ = 7.0) 3.75 bm (2H, >CH -N<), 7.18 bd (1H, -NH-, $J_{H-C-N-H}$ =8.0).

For $C_{29}H_{54}N_2O_2$ (462.76) calculated: 75.27 % C, 11.76 % H, 6.00 % N; found: 75.41 % C, 11.89 % H, 5.64 % N.

Glycerol tri[U-^{14}C]palmitate (IV)

324 MBq of [U-^{14}C]palmitic acid (0.02 mmol) in benzene solution were diluted with 0.42 mmol of non active acid and this solution was evaported in the reaction flask together with 13.8 mg (0.15 mmol) of glycerol and 30 mg (0.27 mmol) of DMAP. The flask was equipped with a magnetic stirrer and an adaptor for the work in inert atmosphere, the flask was flushed with argon and through the silicon septum 5 ml of 1,2-dichloroethane were added. After complete dissolution of reagents the flask was covered with an Al-foil and 0.44 ml of a 0.5 M solution of DCC in benzene were added. After two hours a further 0.22 ml of this solution were added followed by 0.11 ml after 18 hours, then 0.06 ml after another 2 hours and again 0.06 ml after 2 hours i.e. total of 0.89 ml. To convert the last traces of diglyceride to triglyceride 55 mg of palmitic acid were dissolved in 1 ml of 1,2-dichloroethane and the solution was injected into reaction mixture together with 0.3 ml of the

DCC solution. After standing overnight radio-TLC revealed, that 92 % of the activity was in the form of glycerol tri[U-^{14}C]palmitate.

The reaction mixture was evaporated to dryness, the residue was extracted with 5 ml of benzene, then 5 ml of toluene and after concentration to about 2 ml the extract was applied to a flash-silicagel column (25 g) conditioned by n-hexane. The column was eluted by n-hexane-4 % (vol.) ethyl acetate. The fractions of eluate were monitored by radio-TLC, the fractions containing pure glycerol tri[U-^{14}C]palmitate (IV) were combined and evaporated to dryness - 105 mg (86.7 %). This residue was dissolved in 10 ml of toluene and activity of the solution was assayed - 276 MBq (85.2 %). Radiochemical purity checked by radio-TLC was 98.6 %, molar activity (calculated from the total activity and the mass) was 2123 MBq.mmol^{-1}.

Glycerol tri[U-^{14}C]oleate

The esterification of 9.2 mg (0.1 mmol) of glycerol by 189 MBq of [U-^{14}C]oleic acid with molar activity 630 MBq.mmol^{-1} by gradual addition of totaly 0.605 ml of the 0.5M DCC in benzene catalyzed by 18 mg (0.15 mmol) of DMAP in 5 ml of 1,2-dichloroethane followed the protocole for glycerol tri[U-^{14}C] palmitate. DCC solution was added following this pattern: 0.3 ml - 18 hours - 0.15 ml - 6 hours - 0.075 ml - 3 hours - 0.04 ml - 18 hours - 0.04 ml. After finishing the esterification by non active oleic acid (50 mg, 0.3 ml of DCC solution) and after flash-chromatography (n-hexane - 2 % (vol.) ethyl acetate) 78 mg (88 %) of glycerol tri[U-^{14}C]oleate (V) were obtained, the radiochemical yield was 165 MBq (87.5%), Molar activity was thus 1833 MBq.mmol^{-1}, radiochemical purity was 99.4 %.

Glycerol tri[9,10-^{3}H]stearate

To a solution of 336 MBq of [9,10-^{3}H]stearic acid (molar activity 767 MBq.mmol^{-1}), 13.8 mg (0.15 mmol) of glycerol and

22 mg (0.2 mmol) of DMAP in 5 ml of 1,2-dichloroethane, was under stirring under argone atmosphere and exclusion of day light, added totaly 0.605 ml of 0.5M DCC solution in benzene following this pattern: 0.3 ml - 5 hours - 0.15 ml -16 hours - 0.075 ml - 2 hours - 0.04 ml - 2 hours - 0.04 ml. After another 2 hours the reaction was completed by non active stearic acid (50 mg, 0.3 ml of DCC solution) and left for 10 hours. The residue after evaportion of the solvents was extracted with 10 ml of hot benzene and then 10 ml of hot toluene. The combined extracts were evaporated to dryness, dissolved in 2 ml of benzene and the solution was applied to a flash-silicagel column (20 g). After elution (n-hexane - 3 % vol. of ethyl acetate) and fractions monitoring 123 mg (92 %) of glycerol tri[9,10-^{3}H] stearate were obtained with total activity 306 MBq (91 %).
The molar activity was thus 2186 MBq/mmol^{-1}, radiochemical purity was 100 %.

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LITERATURE

1. Talbot J.N., Courtis G., Milhaud G. - J.Fr.Biophys.Med.Nucl. 4:39-43 (1980).
2. Butler R.N. and Gehling N.J. - Clin.Chim.Acta 112:371-74 (1981).
3. Goff J.S. - Gastroenterology 83:44-46 (1982).
4. O´Keefe S.J., Adam J. - S.Afr.Med.J. 66:763-65 (1984).
5. Weinman E.O., Chaikoff I.L., Dauben W.G., Gee M., Entenman G. - J.Biol.Chem. 184:735-44 (1950).

6. Weinman E.O., Chaikoff I.L., Stevens B.P., Dauben W.G. - J.Biol.Chem. 191:523-29 (1951).
7. Scheij R. - Rec.trav.chim. 18:169 (1899).
8. Bellucci, Manzetti - Atti R.Accad.Linzei 20:125 (1911).
9. Bhattacharya A., Hilditch C. - Proc.Roy.Soc. 129A:469 (1930).
10. Clarkson C.E., Malkin T. - J.Chem.Soc. 1934:666-71.
11. Reiser R., Bryson M.J., Carr M.J., Kviken K.A. - J.Biol.Chem. 194:131-38 (1952).
12. Partheil R., Von Velsen A. - Arch.Pharm. (Weinheim) 238:267 (1900).
13. Stephenson M. - Biochem.J. 7:432-35 (1913).
14. Dauben W.G. - J.Amer.Chem.Soc. 70:1376-78 (1948).
15. Rakoff H., Emken E. A. - J.Labelled Compd.Radiopharm. 19:19-33 (1982).
16. Bourne E.J., Stacey M., Tatlow J.C., Tedder J.M. - J.Chem.Soc. 1949:2976-79.
17. Hassner A., Alexanian V. - Tetrahedron Lett. 46:4475-78 (1978).
18. Hassner A., Krepski L.R., Alexanian V. - Tetrahedron 34:2069-76 (1978).
19. Patel K.M., Sklar L.A., Currie R., Pawnall H.J., Morriset J.D., Sparrow J.T. - Lipids 14:816-18 (1979).
20. König W., Geiger R. - Chem.Ber. 103:788 (1970).
21. Formstecher P., Lustenberger P., Dautrevaux M. - Steroids 35:265-71 (1980).
22. Arendt A., Kolodiejczyk A.M. - Tetrahedron Lett. 40:3867-68 (1978).
23. Elbert T. - Czech. 252228.