Synthesis of an unsaturated fatty acid analogue (18-(4′-azido-2′-hydroxybenzoylamino)-oleic acid) and its interaction with lysophosphatidylcholine: acyl-CoA-O-acyltransferase

Lars Gehring,* Dirk Haase,† Kai Habben,* Claus Kerkhoff,* Hartmut H. Meyer,† and Volkhard Kaever[1,*]

Institut für Molekularpharmakologie,* Medizinische Hochschule Hannover, D-30623 Hannover, Germany, and Institut für Organische Chemie,† Universität Hannover, D-30167 Hannover, Germany

Abstract **Acylation/deacylation reactions represent a basic requirement of triglyceride as well as phospholipid metabolism, and maintenance of membrane lipid composition. In order to examine enzymes participating in these pathways, we synthesized 18-(4′-azido-2′-hydroxybenzoylamino)-oleic acid, an iodinable photoaffinity analogue of oleic acid as a new tool for analyzing enzymes, especially those binding unsaturated fatty acids or acyl-CoAs. For the synthesis of ω-amino-oleic acid, coupling two bifunctional C_9-components was used. The described synthesis scheme is also suited for the specific generation of other fatty acid analogues with distinct positions of the double bond. The functionality of 18-(4′-azido-2′-hydroxybenzoylamino)-oleic acid was investigated with the enzyme lysophosphatidylcholine:acyl-CoA-O-acyltransferase (LAT) [EC 2.3.1.23], an enzyme that shows high specificity towards (poly)unsaturated fatty acyl-CoAs. It could be shown that the photolabel, esterified with coenzyme A, acts in the dark as a reversible inhibitor of the enzyme activity, but photolysis of the label results in irreversible inactivation of LAT. This inactivation could be prevented by addition of the native substrate arachidonyl-CoA during photolysis. Several proteins could be specifically visualized using the iodinated analogue. The data indicate that this new photoaffinity label may have application to identify and characterize lipid biosynthetic enzymes using unsaturated fatty acids as well as acyl-CoA binding proteins and the active site of these proteins.—**Gehring, L., D. Haase, K. Habben, C. Kerkhoff, H. H. Meyer, and V. Kaever. **Synthesis of an unsaturated fatty acid analogue (18-(4′-azido-2′-hydroxybenzoylamino)-oleic acid) and its interaction with lysophosphatidylcholine:acyl-CoA-O-acyltransferase. *J. Lipid Res.* 1998.** 39: **1118–1126.**

Supplementary key words 18-(4′-azido-2′-hydroxybenzoylamino)-oleoyl-CoA • ω-amino-oleic acid • photoaffinity label • acyl-CoA analogue

Fatty acids and especially their activated derivatives fatty acyl-CoAs play a fundamental role in carbohydrate and fatty acid metabolism as well as in the biosynthesis of phospholipids and triglycerides. They are also substrates for a number of enzymes that transfer fatty acids to proteins (1). Additionally, fatty acyl-CoAs have been found to be necessary for the budding of transport vesicles from the Golgi apparatus (2).

Acyl-CoA acts as a primary substrate for various membrane-associated proteins catalyzing lipid biosynthesis. These acyltransferases play key roles in lipid biosynthesis. The de novo biosynthesis of glycerolipids starts with the acylation of glycerol-3-phosphate to form lysophosphatidic acid (LPA). The acylation of LPA is catalyzed by lysophosphatidic acid:acyl-CoA-O-acyltransferase to form phosphatidic acid, the precursor for anionic phospholipids and diacylglycerol. In addition to the de novo biosynthetic enzyme activities, microsomal membranes show high lysophospholipid:acyl-CoA-O-acyltransferase (LPLAT) activity. These enzymes catalyze the conversion of lysophospholipids to phospholipids by direct acylation. Within this mechanism, the LPLATs constitute an important part of a deacylation–reacylation cycle (Lands cycle (3)) that is one primary route for rapidly altering the fatty acid composition of phospholipids. By this mechanism the membrane lipid composition is maintained (3–6) and the level of unesterified free arachidonic acid (7, 8) regulating the synthesis of leukotrienes and prostaglandins (9) is controlled. Additionally, the LPLATs are thought to play a crucial role in the early phase of T-lymphocyte activation

Abbreviations: ASO-CoA, 18-(4′-azido-2′-hydroxybenzoylamino)-oleoyl-CoA; CoA, Coenzyme A; DMF, dimethylformamide; DMPU, 1,3-dimethyltetrahydro-2(1H)-pyrimidinone; DMSO, dimethyl sulfoxide; KHMDS, potassium hexamethyldisilazide; LAT, lysophosphatidylcholine:acyl-CoA-O-acyltransferase; LPA, lysophosphatidic acid; LPLAT, lysophospholipid:acyl-CoA-O-acyltransferase; LPC, 1-palmitoyl-lysophosphatidylcholine; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; PE, petrolether; THP, tetrahydropyran.

[1]To whom correspondence should be addressed.

by catalyzing the elevated incorporation of polyunsaturated fatty acids into plasma membrane phospholipids (10–15).

Whereas the other enzyme(s) participating within the Lands cycle, especially the phospholipases A_2 (PLA_2), are quite well examined (16), the number and properties of the enzymes representing LPLAT activity are not well understood. It has been reported that distinct acyltransferases with different specificities for the lysophospholipid acyl acceptor exist (17, 18). Other evidence suggests that an LPC-specific LPLAT enzyme (LAT) (EC 2.3.1.23) may exist in separate forms differing in specificity for unsaturated and saturated fatty acyl-CoAs. For example, Lands et al. (7) reported that liver microsomal acyltransferase shows a high specificity for unsaturated acyl-CoA, whereas in lung microsomes it prefers palmitoyl-CoA compared to oleoyl-CoA as substrate (19).

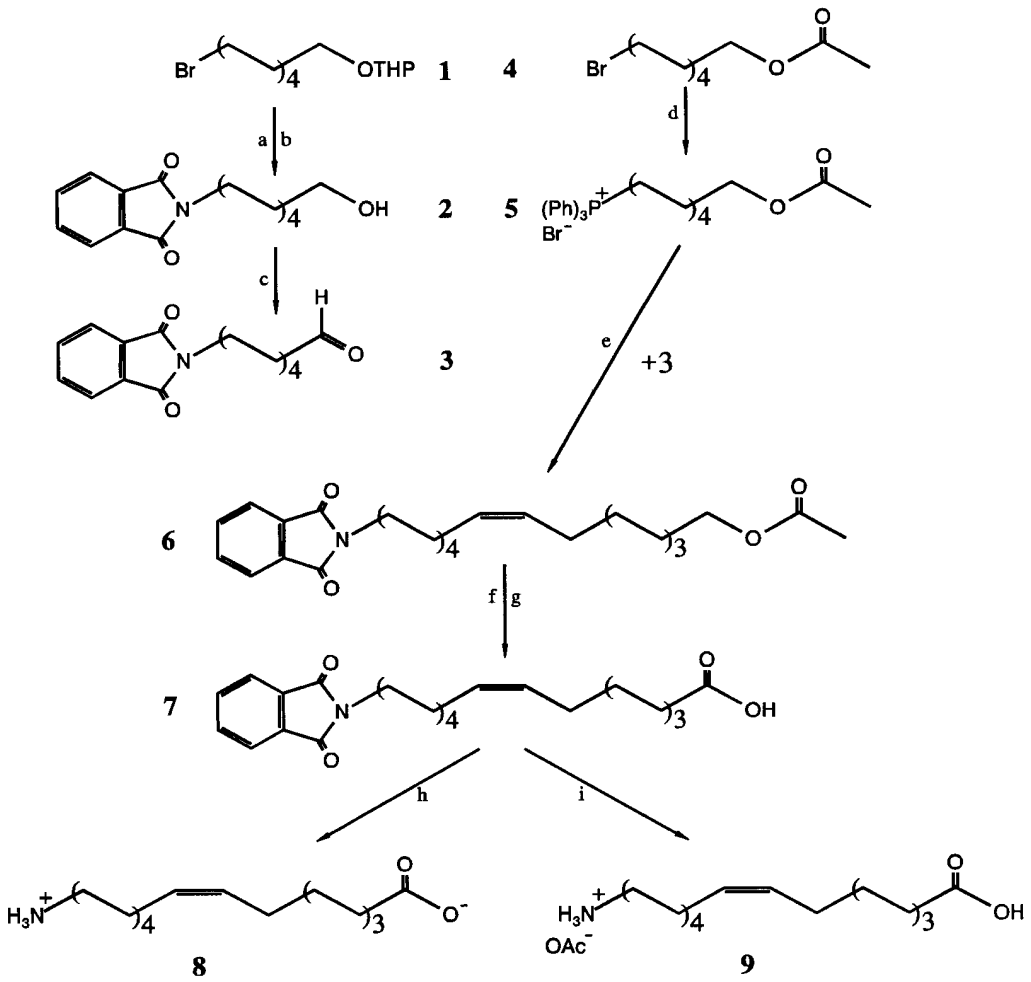
Several attempts have been made to purify and characterize LPLATs (20–23) either using the capability of the enzymes for generating a microsomal environment by themselves (20, 21) or by a sequential combination of different chromatographic separation methods (22, 23). Until now, a high degree of purification and molecular cloning have not been reported.

An additional possibility to characterize enzymes is the use of photoaffinity probes. Several attempts to analyze acyl-CoA binding proteins have been made using a photoaffinity analogue of a medium-chain saturated fatty acid (24–30). Because of the high specificity of LAT in human placenta and pig spleen (31) that we examined, the synthesis of an unsaturated fatty acid (oleic acid) analogue with facilities for ω-terminal coupling with a (iodinable) photoaffinity label has become a highly desirable goal. In this paper, the synthesis of a photoaffinity oleoyl-CoA (ASO-CoA) and its interaction with the enzyme LAT is described for the first time.

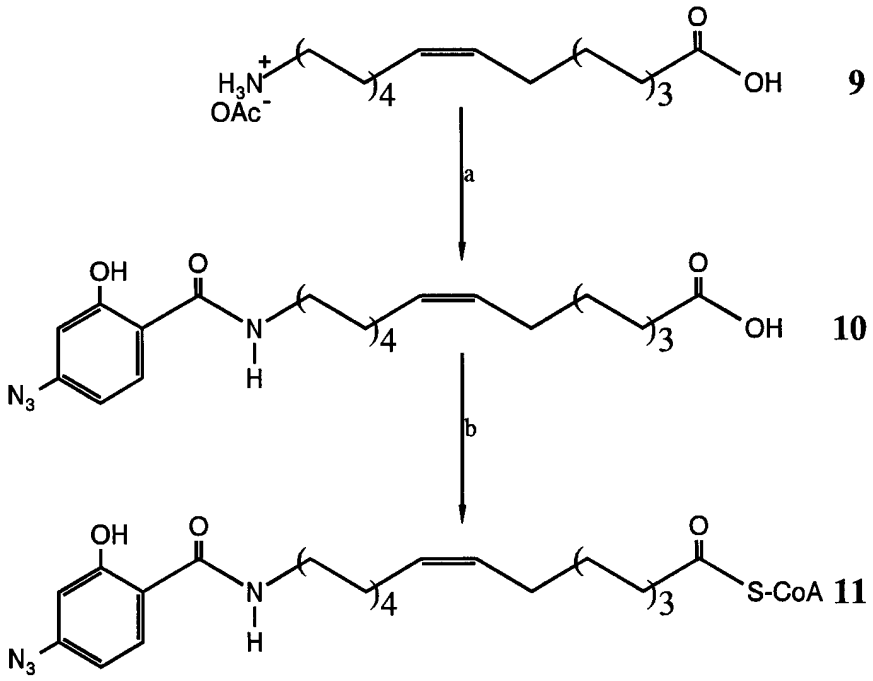
MATERIALS AND METHODS

Synthesis scheme

For the synthesis of the ω-amino-oleic acid **8/9**, a flexible synthesis concept of coupling two bifunctional C_9-components was used (**Scheme 1**).



Scheme 1. Reagents: (a) K-phthalimide, DMF; (b) CH_3OH, HCl; (c) oxalyl chloride, DMSO, $N(Et)_3$; (d) triphenylphosphine, CH_3CN; (e) DMPU, KHMDS; (f) CH_3OH, CH_3ONa; (g) CrO_3, acetone; (h) $N_2H_4 \cdot H_2O$, C_2H_5OH, HCl, NaOH, HCl; (i) $N_2H_4 \cdot H_2O$, C_2H_5OH, HCl, NaOH, HOAc.



Scheme 2. Reagents: (a) NHS-ASA, pyridine, dichloromethane; (b) oxalyl chloride, dichloromethane, DMF_{cat}, CoASH, THF, H_2O, NaOH, $HClO_4$, diethylether.

The starting point of this synthesis was 1,9-nonanediol which was converted to 1-bromo-9-(tetrahydropryran-2′-yl-oxy)-nonane (**1**) (32) and 9-bromo-nonylacetate (**4**) (33). The exchange of the halogen of **1** against a phthalimido function and the deprotection of the alcohol gave 9-phthalimido-nonanol (**2**), carrying a protected amino function. Oxidation of the alcohol resulted in 9-phthalimido-nonanal (**3**). The second component for the Wittig reaction, 9-acetoxy-nonyl-triphenylphosphonium bromide (**5**) (34), was generated by reaction of **4** with triphenylphosphine.

To ensure the *Z*-double bond of **9**, the coupling of the aldehyde **3** with the phosphorylide from **5** was carried out using the *Z*-specific variant of the Wittig-reaction (35, 36). Using a mixture of THF and DMPU as solvent and potassium hexamethyldisilazide as the base, pure (*Z*)-18-phthalimido-9-octadecenyl acetate (**6**) was formed. Deacetylation of **6** followed by oxidation with the Jones reagent (chromic acid) gave the phthalimido derivative **7** of the ω-amino-oleic acid which was deprotected via hydrazinolysis and subsequent acidic and basic hydrolysis. The free amino acid **8** was obtained by precipitation at pH 5–6. Alternatively, the final addition of acetic acid to the reaction mixture gave the acetate **9** after chromatographic purification. No trace of the *E*-isomer was detectable in the ^{1}H- and ^{13}C-NMR spectra of **6** and the compounds derived from **6** in the following steps of the synthesis.

The following reactions (**Scheme 2**) leading to the photolabile coenzyme A ester were totally carried out in the dark (red light). Under this condition the acylation of **9** with *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) was carried out in heterogeneous phase to obtain **10**. The addition of three equivalents of oxalyl chloride to **10** gave the corresponding acyl chloride which was immediately coupled with coenzyme A leading to the photoaffinity analogue of oleoyl-CoA **11** (ASO-CoA).

General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker WP200 and AM400 spectrometer. Chemical shifts were reported in δ (ppm) relative to tetramethylsilane. All coupling constants were described in Hz. Mass spectral data were obtained on Finnigan MAT312. Unless otherwise stated, the reaction mixture was worked up with water and a suitable solvent; the aqueous phase was extracted again; and the organic solvents were pooled, washed with saturated NaCl solution, dried with $MgSO_4$, and evaporated in vacuo. Flash column chromatography was performed on silica gel (50–200 μm) under a pressure of about 4 psi. All solvents were obtained from commercial suppliers and distilled and dried if necessary before use. THF, CH_3CN, CH_2Cl_2, DMPU, DMF, and acetone were stored over a molecular sieve. Pyridine and triethylamine were distilled and stored over potassium hydroxide. Petrolether represents a mixture of medium-chain alkanes with a boiling range of 35°–70°C. Coenzyme A was obtained from Sigma and [^{14}C]-1-palmitoyl-lysophosphatidylcholine (spec. act. 40–60 mCi/mmol) was purchased from NEN.

1-Bromo-9-(tetrahydropyran-2′-yl-oxy)-nonane (1)

Substance **1** was synthesized as described by Butenand et al. (32), based on 1,9-nonanediol.

9-Phthalimidononanol (2)

In a round-bottom flask fitted with magnetic stirring bar 20.0 g (65.1 mmol) **1** and 15.7 g (84.5 mmol) potassium phthalimide were dissolved in 40 mL DMF and heated at 70°C for 4 h. The mixture was extracted with water and CH_2Cl_2. The organic phase was dried with $MgSO_4$ and evaporated in vacuo, resulting in a pale yellow oil. It was dissolved in 500 mL methanol and 10 mL 6 N HCl and heated to reflux for 1 h. After cooling to room temperature, the solution was neutralized with an aqueous solution of $NaHCO_3$. The methanol was evaporated and the residue was extracted with CH_2Cl_2 and water. The organic phase was dried, the residue was purified by flash column chromatography (diethylether) to give **2** as white crystals (16.9 g, 89.7%): ^{1}H NMR ($CDCl_3$) δ 7.83 (m, 2H), 7.71 (m, 2H), 3.67 (t, 2H, J = 7Hz for NCH_2), 3.63 (t, 2H, J = 7Hz for CH_2O), 2.24 (s, 1H, for OH), 1.78–1.48 (2m, 4H, for $2CH_2$), 1.45–1.20 (m, 10 H, for $5CH_2$);

^{13}C NMR ($CDCl_3$) δ 165.50, 133.89, 132.13, 123.16, 62.84, 38.02, 32.73, 29.40, 29.30, 29.05, 28.55, 26.79, 25.70; MS (90°C) *m*/*z* 289 (M^+, 19), 259 (8), 202 (4), 188 (5), 175 (7), 174 (11), 161 (97), 160 (100), 148 (27), 133 (16), 130 (22), 117 (6), 105 (15), 104 (14), 77 (20); ($C_{17}H_{22}NO_3$).

9-Phthalimidononanal (3)

To a solution of 5.6 mL (61.6 mmol) oxalyl chloride in 85 mL CH_2Cl_2 at a temperature of −65° to −70°C, 8.8 mL DMSO was added within 15 min; the solution was stirred for further 30 min. A solution of 16.7 g (57.7 mmol) **2** in 25 mL CH_2Cl_2 was added dropwise and the mixture was stirred for additional 30 min. After the addition of 38.8 mL triethylamine, the solution was warmed to room temperature within 1 h and washed consecutively with water, diluted HCl, aqueous solution of $NaHCO_3$, and water. Purification by flash column chromatography (E:PE = 1:1) afforded **3** as white crystals (14.2 g, 85.6%). ^{1}H NMR ($CDCl_3$) δ 9.76 (t, 1H, *J* = 1.8 Hz for CHO), 7.84 (m, 2H), 7.71 (m, 2H), 3.68 (t, 2H, *J* = 7Hz for NCH_2), 2.42 (dt, 2H, *J* = 2Hz, *J* = 7Hz for CH_2O), 1.78–1.55 (2m, 4H, for $2CH_2$), 1.45–1.25 (m, 8H, for $4CH_2$); ^{13}C NMR ($CDCl_3$) δ 202.99, 168.20, 133.67, 131.96, 122.93, 43.65, 37.76, 28.97, 28.84, 28.73, 28.33, 26.54, 21.82; MS (70°C) *m*/*z* 287 (M^+, 4), 259 (13), 244 (7), 202 (5), 188 (6), 174 (11), 161 (41), 160 (100), 148 (18), 133 (16), 130 (21), 105 (14), 104 (14), 77 (22); ($C_{17}H_{21}NO_3$).

9-Bromononyl acetate (4)

Substance **4** was synthesized as described by Goto et al. (33), based on 1,9-nonanediol.

9-Acetoxynonyltriphenylphosphonium bromide (5)

A solution of 7.7 g (29.0 mmol) **4** and 7.0 g (26.7 mmol) triphenylphosphine in acetonitrile was heated to reflux for 18 h within a nitrogen atmosphere (34). The solvent was evaporated, 50 mL hexane was added to the stirred oily residue, and the resulting emulsion was heated to reflux for 10 min. The stirring was stopped and the resulting lower phase containing **5** was cooled down quickly to 0° to −5°C; the yet warm hexane upper phase was decanted. This purification procedure was repeated twice with hexane, with ethyl acetate, and again with hexane. Traces of the solvents were eliminated by heating at 60°C for several hours in vacuo. Substance **5** was obtained as a viscous pale oil (11.9 g, 84.6%). ^{1}H NMR ($CDCl_3$) δ 7.95–7.65 (m, 15H), 4.02 (t, 2H, *J* = 7Hz for CH_2O), 3.80 (m, 2H, for CH_2P), 2.05 (s, 3H, for CH_3), 1.75–1.45 (m, 4H, for $2CH_2$), 1.35–1.15 (m, 10 H, for $5CH_2$).

(Z)-18-phthalimido-9-octadecenyl acetate (6)

In a round-bottom flask fitted with a magnetic stirring bar, 6.9 g (13.1 mmol) **5** was dissolved in 40 mL dried THF and 4 mL DMPU under nitrogen atmosphere. At a temperature of −30°C, 21.7 mL of a solution containing 15% of KHMDS (14.3 mmol) in toluene was added dropwise within 30 min resulting in an orange reaction mixture. At the same temperature, a solution of 3.7 g (12.9 mmol) **3** in THF was added and the pale yellow mixture was stirred overnight at room temperature. Extraction was performed using dilute sulfuric acid and diethylether. Purification by flash column chromatography (E:PE = 1:6–1:1) led to **6** as a waxy solid (5.4 g, 92%). ^{1}H NMR ($CDCl_3$) δ 7.84 (m, 2H), 7.80 (m, 2H), 5.33 (m, 2H, for 2CH), 4.05 (t, 2H, *J* = 7Hz for CH_2O), 3.67 (t, 2H, *J* = 7Hz for NCH_2), 2.05 (s, 3H, for CH_3), 2.00 (m, 4H, for $2CH_2$), 1.65 (m, 4H, for $2CH_2$), 1.5–1.2 (m, 20H, for $10CH_2$); ^{13}C NMR ($CDCl_3$) δ 171.11, 168.33, 133.72, 132.08, 125.78, 123.04, 64.54, 37.95, 29.62, 29.30, 29.11, 28.50, 27.09, 26.76, 25.81, 20.92; MS (130°C) *m*/*z* 455 (M^+, 10), 396 (4), 313 (7), 300 (5), 244 (3), 188 (4), 174 (7), 160 (100), 148 (21), 133 (7), 130 (14), 121 (10), 110 (9), 109 (15), 95 (29), 81 (33); ($C_{28}H_{41}NO_4$).

(Z)-18-phthalimido-9-octadecenic acid (7)

The solution of 5 g (11 mmol) **6** in 80 mL dried methanol was adjusted to pH 9 with sodium hydride. The mixture was stirred for 1 h at room temperature and neutralized with acetic acid and the volatile evaporated. The crude product was purified using flash chromatography (E:PE = 1:1), giving (*Z*)-18-phthalimido-9-octadecenol as a waxy solid (4.2 g, 92%). ^{1}H NMR ($CDCl_3$) δ 7.83 (m, 2H), 7.71 (m, 2H), 5.33 (m, 2H, for 2CH), 3.67 (t, 2H, *J* = 7Hz for NCH_2), 3.63 (t, 2H, *J* = 7Hz for CH_2O), 2.28 (s, 1H, for OH), 2.00 (m, 4H, for $2CH_2$), 1.75–1.20 (m, 24H, for $12CH_2$); ^{13}C NMR ($CDCl_3$) δ 168.47, 133.82, 132.14, 129.84, 123.13, 62.99, 38.05, 32.79, 29.70, 29.66, 29.47, 29.33, 29.35, 29.19, 29.16, 29.15, 28.58, 27.14, 26.83, 25.73. This alcohol (10.1 mmol) was dissolved in 100 mL acetone at 0°C and 8.4 mL of Jones reagent (27.0 g chromium trioxide and 23 mL conc. sulfuric acid dissolved in water in a final volume of 100 mL) was added. After 10 min, 2-propanol was added to eliminate excess chromic acid and the mixture was diluted with 10 mL water and the acetone was evaporated in vacuo. The suspension was extracted with diethylether; purification by flash column chromatography (E:PE = 1:6–3:1) led to **7** as a waxy solid. (4.2 g, 80.5%). ^{1}H NMR ($CDCl_3$) δ 7.85 (m, 2H), 7.70 (m, 2H), 5.53 (m, 2H, for 2CH), 3.67 (t, 2H, *J* = 7Hz for NCH_2), 2.35 (t, 2H, *J* = 7Hz for CH_2COO), 2.00 (m, 4H, for $2CH_2$), 1.65 (m, 4H, for $2CH_2$), 1.40–1.15 (m, 18H, for $9CH_2$); ^{13}C NMR ($CDCl_3$) δ 179.99, 168.46, 133.83, 132.15, 129.90, 129.75, 123.15, 38.05, 34.07, 29.69, 29.66, 29.38, 29.19, 29.16, 29.14, 29.05, 29.03, 28.58, 27.17, 27.15, 26.85, 24.67; MS (170°C) *m*/*z* 427 (M^+,8), 409 (70), 391 (50), 262 (24), 244 (12), 234 (17), 230 (10), 216 (12), 202 (13), 188(18), 174 (33), 165 (16), 161 (72), 160 (100), 151 (30), 148 (74), 135 (32), 130 (72), 121 (28), 109 (44), 104 (44), 98 (53), 95 (73), 81 (77), 68 (77); ($C_{26}H_{37}NO_4$).

(Z)-18-amino-9-octadecenic acid (8)

The solution of 2.14 g (5.0 mmol) **7** and 1.0 g (20.0 mmol) hydrazine hydrate in 25 mL ethanol was heated to reflux for 15 min until the product sedimented and the flask contents became solid. Heating was continued for additional 2 h, the hydrazine hydrate and the solvent were evaporated in vacuo; the dry residue was dissolved in 20 mL ethanol and 5 mL 2 N HCl giving a pH value of about 1–2 and the mixture was heated to reflux for 3 h. About 10 mL of ethanol was removed by distillation; the suspension was cooled down to room temperature and the precipitated phthalimide was removed by filtration. Four mL of 6 N NaOH was added to the filtrate and heating to 80°C was continued for 30 min until the ethylester of **8** (R_f = 0.5, E:PE 1:1) was totally hydrolyzed. After cooling down to room temperature, the mixture was neutralized with 2 N HCl; the remaining ethanol was removed by distillation. The suspension was cooled down to 5°C and filtered. **8** was obtained as a white solid (1.33 g, 89.4%). ^{1}H NMR ($CDCl_3$/CF_3COOD) δ 5.45–5.25 (m, 2H, for 2CH), 2.90 (t, 2H, *J* = 7Hz for NCH_2), 2.28 (t, 2H, *J* = 7Hz for CH_2COO), 2.12–1.95 (m, 2H, for CH_2), 1.78–1.50 (m, 4H, for $2CH_2$), 1.48–1.15 (m, 20H, for $10CH_2$); ^{13}C NMR (CD_3OD/CF_3COOH) δ 180.33, 133.36, 43.27, 37.53, 33.35, 33.30, 32.92, 32.83, 32.77, 32.76, 32.69, 31.08, 30.66, 30.63, 29.99, 28.62; ($C_{18}H_{35}NO_2$).

(Z)-18-amino-9-octadecenic acid acetate (9)

In the same manner as described for the synthesis of **8**, 2.14 g (5.0 mmol) **7** were processed. In difference, the final neutralization step was performed with excess acetic acid instead of 2 N HCl. All solvents were evaporated in vacuo and the residue was mixed with an equal amount of silica gel. Flash column chroma-

tography was performed with CH_2Cl_2: methanol = 9:1 and CH_2Cl_2: methanol: acetic acid = 9:1:1. Evaporation of the solvents in vacuo for several days at room temperature yielded **9** (1.60 g, 89.5%). ^{1}H NMR ($CDCl_3$/CF_3COOD) δ 5.47–5.23 (m, 2H, for 2CH), 2.91 (t, 2H, J = 7Hz for NCH_2), 2.28 (t, 2H, J = 7Hz for CH_2COO), 2.10–1.97 (m, 2H, for CH_2), 2.00 (s, 3H, for CH_3), 1.77–1.50 (m, 4H, for $2CH_2$), 1.48–1.22 (m, 20H, for $10CH_2$). The ^{13}C NMR spectrum (CD_3OD/CF_3COOH) is identical compared to that of compound **8**. It only contains additional signals derived from acetic acid (δ 177.65, 23.55).

(Z)-18-(4′-azido-2′-hydroxybenzoylamino)-9-octadecenic acid (10)

All following procedures were carried out totally in the dark (red light). Substance **9** (128.5 mg, 0.36 mmol) was suspended in 5 mL dried THF (100 mg, 0.36 mmol). *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) dissolved in 3 mL THF and 1 mL pyridine were added. The suspension was stirred for several days, the reaction course was monitored by thin-layer chromatography ($CHCl_3$: methanol = 5:1; R_f(educt) = 0.1; R_f(**10**) = 0.6). The solvents were evaporated in vacuo; the residue was resuspended in 4 mL THF and the pH was adjusted to 5 with potassium hydrogensulfate. After distillation of THF, the aqueous suspension was mixed with silica gel and separated by flash column chromatography ($CHCl_3$: methanol = 10:1). Substance **10** was obtained as a waxy solid (130.5 mg, 79.1%). ^{1}H NMR ($CDCl_3$) δ 7.35 (d, 1H, J = 8Hz), 6.66 (d, 1H, J = 2Hz), 6.49 (dd, 1H, J = 8Hz, J = 2Hz), 6.4 (bs, 1H, for NH), 5.45–5.26 (m, 2H, for 2CH), 3.51–3.33 (m, 2H, for NCH_2), 2.32 (t, 2H, J = 7Hz, for CH_2COO), 2.10–1.92 (m, 4H, for $2CH_2$), 1.73–1.50 (m, 4H, for $2CH_2$), 1.42–1.22 (m, 18H, for $9CH_2$); ^{13}C NMR ($CDCl_3$) δ 179.42, 169.29, 162.88, 145.70, 129.88, 129.85, 126.90, 111.26, 109.82, 108.11, 39.65, 33.99, 29.68, 29.63, 28.46, 29.41, 29.26, 29.17, 29.13, 29.03, 27.15, 26.96, 24.68; ($C_{25}H_{38}N_4O_4$).

(Z)-18-(4′-azido-2′-hydroxybenzoylamino)-9-octadecenyl-CoA (11) (ASO-CoA)

All following procedures were carried out totally in the dark (red light). Substance **10** (81.0 mg, 0.176 mmol) was suspended in 3 mL dried ice-cold CH_2Cl_2 (37) and the temperature was kept at 0°C. A catalytic amount of 1.6 mg (0.022 mmol) DMF in 0.1 mL CH_2Cl_2 and 64.1 mg (0.5 mmol) oxalyl chloride in 0.52 mL CH_2Cl_2 were added. After stirring for 30 min at room temperature, a clear solution was achieved. The solution was kept at room temperature for further 2 h; the solvent was evaporated in vacuo. (*Z*)-18-(4′-azido-2′-hydroxybenzoylamino)-9-octadecenyl chloride was obtained as a partially crystalline solid (80.1 mg, 94.9%). The acyl chloride was dissolved in 0.5 mL THF and added in portions to a mixture of 35 mL THF and 15 mL water containing 100 mg (0.13 mmol) coenzyme A within a nitrogen atmosphere. The pH was kept at 8.0 with 1 N NaOH during the addition of the acyl chloride. The reaction was terminated by adding 10% (v/v) $HClO_4$ to give a final pH of about 4. THF was evaporated and the synthesized fatty acyl-CoA was precipitated by cooling to 4°C and addition of 1.5 ml 10% (v/v) $HClO_4$. The precipitate was pelleted by centrifugation at 20,000 *g* for 15 min, washed twice with diethylether, and redissolved in 10 mm Tris-buffer, pH 7.4, containing 150 mm NaCl and 1 mm EDTA (about 80% yield, two steps). The purity was determined by thin-layer chromatography, using RP-18 silica gel plates with n-butanol–water–acetic acid 40:30:20 (v/v/v; R_f = 0.82). No further products could be detected.

Iodination of ASO-CoA

Iodination of ASO-CoA was performed by using chloramine T (38). In brief, 50 nmol of ASO-CoA was dissolved in 0.5 m sodium phosphate buffer (pH 7.0) containing 16 nmol $Na_2S_2O_5$ and 1 mCi Na[^{125}I] in 0.1 m NaOH in a total volume of 200 μL. The reaction was started by adding 90 nmol chloramine T in 20 μL buffer. After incubation for 30 min, 315 nmol $Na_2S_2O_5$ in 50 μL buffer was added. After addition of 300 μL NaCl solution (10%), the iodinated product was extracted with ethyl acetate and stored at −20°C.

Preparation of crude membranes and partial purification of LAT activity

Crude membranes were prepared from human placenta by differential centrifugation similar to the method described by Kerkhoff et al. (31). Briefly, the placenta (350 g) was washed with ice-cold LAT assay buffer (10 mm Tris, pH 7.4, containing 150 mm NaCl and 1 mm EDTA), minced, and homogenized in 500 mL buffer using a glass/Teflon homogenizer. The homogenate was filtered through a wire mesh, sonicated at 4°C for 10 min, and centrifuged at 15,000 *g* for 60 min at 4°C. The supernatant was centrifuged at 200,000 *g* for 2 h at 4°C. The pellet was washed and resuspended in LAT storage buffer (50 mm Tris, pH 7.4, 250 mm sucrose, 1 mm EDTA, and 20% (v/v) ethyleneglycol), prior to use to a protein concentration of about 50 mg/mL and stored frozen at −80°C. Upon storage for several weeks, no loss of the original LAT activity was observed.

Crude membranes containing 10 mg/mL of protein (final) were treated with LAT assay buffer containing 1.5 m NaCl for 30 min at 4°C. After centrifugation at 100,000 *g* for 60 min at 4°C, ammonium sulfate was added to the supernatant to a final concentration of 40% (w/v). The solution was stirred for 30 min and centrifuged at 20,000 *g* for 30 min. The pellet was dissolved in 10 mm potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight.

Further purification was achieved using hydroxyapatite column chromatography. The dialyzed sample was applied to the column equilibrated with the above-mentioned phosphate buffer. The column was washed with 200 mm potassium phosphate buffer, pH 7.0, and the enzyme activity was eluted applying a gradient up to 500 mm potassium phosphate, thereby maintaining an additional concentration of 1 m potassium chloride. Fractions containing enzyme activity were pooled, supplemented with 10% glycerol (w/v), and stored at −80°C.

Concentration of proteins

Concentration of proteins prior to SDS-PAGE was performed as described in detail by Wessel and Flügge (39).

LAT enzyme activity assay

The lysophosphatidylcholine:acyl-CoA-O-acyltransferase activity was measured by the formation of [^{14}C]phosphatidylcholine (PC) using arachidonyl-CoA and [^{14}C]-1-palmitoyl-lysophosphatidylcholine (LPC) as substrates. The assay was carried out as described by Kerkhoff et al. (31). In brief, the enzyme was incubated with 50 μm [^{14}C]LPC (50,000 dpm) in the presence of 30 μm arachidonyl-CoA. The reaction mixture (0.2 mL final volume) contained Tris-HCl (10 mm, pH 7.4), NaCl (150 mm), and EDTA (1 mm). After incubation for 20 min at 37°C, the reaction was stopped by adding 1.5 mL of $CHCl_3$:CH_3OH 1:2 and 0.2 mL of water. A further addition of 0.5 mL of $CHCl_3$ and 0.5 mL of water was made to separate the phases. After mixing and centrifugation, the $CHCl_3$ layer containing the phospholipids was dried; the residue was dissolved in 10 μL $CHCl_3$ containing 1 nmol of phosphatidylcholine as a marker and applied on a TLC plate. The chromatography was performed using a solution of $CHCl_3$: CH_3OH: HOAc: H_2O 50:25:8:2.5 (v/v). The lipids were stained with Coomassie Brilliant Blue stain according to Nakamura and Handa (40). Educt and product of the LAT reaction were

scraped off the plates and transferred to scintillation vials containing 10 mL of scintillation cocktail. Radioactivity was determined in a LKB 1214 Rackbeta counter. Enzyme activity is given as nmol [^{14}C]PC formed per 20 min.

Studies of the effect of ASA-CoA on enzyme activity in the dark were carried out by using different concentrations of ASA-CoA in the presence or absence of the competing substrate arachidonyl-CoA.

Photolysis

Photolysis was carried out by placing the reaction tubes directly onto a desk-integrated UV lamp (5,000 μW/cm^2; 312 nm)

RESULTS

The ability of ASO-CoA **11** to affect LAT activity in vitro was investigated by using partially purified LAT from human placenta. The enzyme (5 μg) was incubated with 50 μM [^{14}C]LPC (50,000 dpm) and various concentrations of ASO-CoA (0–800 μm) in the presence or absence of 30 μm arachidonyl-CoA. It should be noted that the reaction was completely carried out in the dark.

Figure 1 documents the obtained results. The photolabel was not accepted as a substrate for the enzyme (data not shown) but even without photolysis it acted as an inhibitor of the enzyme activity. The concentration of ASO-CoA at which LAT activity was inhibited by 50% in the presence of arachidonyl-CoA (30 μm) appeared to be about 250 μm.

To rule out any non-specific detergent effects causing inhibition of the enzyme, we tested the effect of both non-specific detergents (CHAPS and octylglucopyranoside) and several fatty acids and fatty acid analogues (oleic acid, oleoyl-CoA, and pre-irradiated ASO-CoA) in concentrations of 400 μm compared to 400 μm ASO-CoA.

Table 1 documents the results. The enzyme activity was reduced to 27% of control LAT activity by 400 μm ASO-CoA. Using pre-irradiated ASO-CoA, the inhibition of enzyme activity was also reduced (66.2% of control). Whereas oleic acid was found to inhibit the enzyme activity comparable to ASO-CoA, oleoyl-CoA induced a strong inhibition of the enzyme. In contrast, the non-specific detergents CHAPS and octylglucopyranoside reduced enzyme activity only slightly (Table 1).

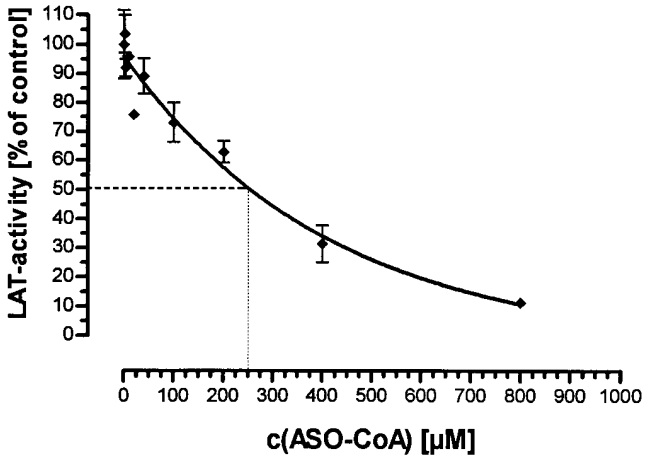


Fig. 1. Effect of ASO-CoA on the LAT reaction. The reaction mixture contained 5 μg microsomal proteins, 50 μm [^{14}C]LPC, 30 μm arachidonyl-CoA, and increasing concentrations of ASO-CoA as indicated. The reaction was totally carried out in the dark. Mean values ± SD from triplicates are presented.

TABLE 1. Comparison of the effect of ASO-CoA and other amphiphilic substances on the LAT reaction

	LAT Activity
	% of control
ASO-CoA	27.0 ± 7.5
ASO-CoA (pre-irridiated)	66.2 ± 9.9
Oleic acid	25.9 ± 5.8
Oleoyl-CoA	5.5 ± 2.6
CHAPS	78.9 ± 1.7
Octylglucopyranoside	84.8 ± 10.0

The reaction mixture contained 5 μg microsomal proteins, 50 μm [^{14}C]LPC, 30 μm arachidonyl-CoA, and 400 μm of the indicated substances. The reaction was totally carried out in the dark. Mean values ± SD from triplicates are presented.

Furthermore, the analogue was characterized for its ability to act as a photoaffinity marker for covalent binding to LAT as a model for unsaturated acyl-CoA utilizing enzymes. Therefore, we coincubated the partially purified enzyme (20 μg) with 250 μm ASO-CoA and increasing concentrations of arachidonyl-CoA in the range of 0–100 μm in a final volume of 0.3 mL in the dark. The samples were incubated on ice for 10 min followed by incubation at 37°C for 3 min to ensure binding equilibration. After UV-irradiation, the solution was dialyzed against the assay buffer for several days. Control samples were also UV-exposed in the absence of photolabel and dialyzed. The measurement of enzyme activity was performed as described in Materials and Methods. As shown in **Fig. 2**, the photolabel reduced the enzyme activity to 5% of control after UV exposure in the absence arachidonyl-CoA. When the UV exposure was carried out with samples containing ASO-CoA and additional arachidonyl-CoA, a diminished inhibition was observed. Using 100 μm arachidonyl-CoA, the enzyme activity was almost completely (about 95%) restored. When the incubation with ASO-CoA without arachidonyl-CoA and the subsequent dialysis was completely carried out in the dark (control II), the enzyme activity was only slightly affected (about 85% of control LAT activity).

The cross-linking efficiency was determined by cross-linking 20 μg protein with iodinated ASO-CoA, concentrating the protein, and measuring the bound radioactivity in relation to the radioactivity used. The efficiency was calculated to be about 15%.

To investigate whether ASO-CoA might identify possible candidate proteins for the LAT, we used the iodinated ASO-CoA for photoaffinity labelling of proteins in SDS-gels. Therefore, we incubated partially purified protein (20 μg) with 1 μCi ^{125}I-labeled ASO-CoA in the presence or absence of 100 μm arachidonyl-CoA for 10 min at 0°C and for 3 min at 37°C. After photolysis, we separated the proteins by SDS-polyacrylamide gel electrophoresis. After

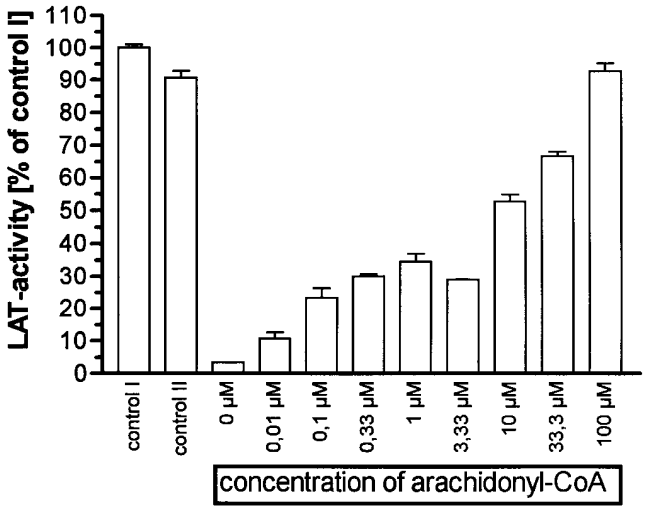


Fig. 2. Reversal of ASO-CoA induced photoinactivation of the LAT enzyme activity by increasing amounts of arachidonyl-CoA. Microsomal proteins (20 µg) were coincubated with 250 µm ASO-CoA and increasing amounts of arachidonyl-CoA as indicated. After UV exposure and dialysis, the LAT activity was determined. Control I represents the incubation of microsomal proteins without ASO-CoA; control II represents the incubation of microsomal proteins with ASO-CoA without UV exposure and subsequent dialysis. Mean values ± SD from triplicates are presented.

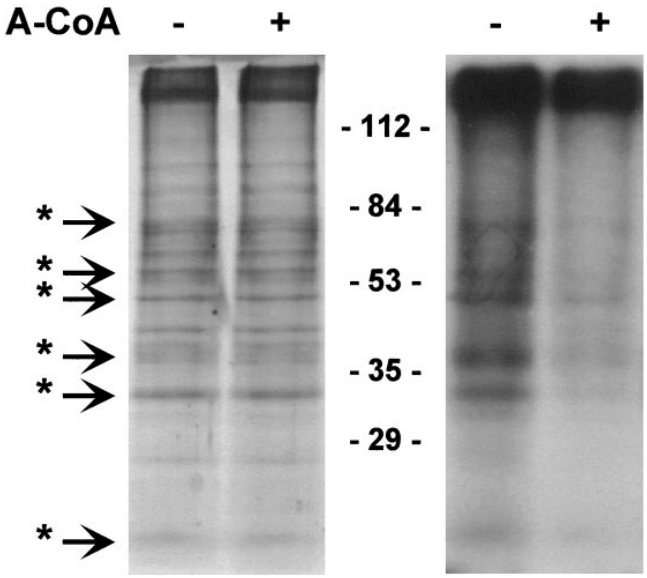


Fig. 3. Visualizing of acyl-CoA binding proteins, i.e., putative candidate proteins for LAT, with ^{125}I-labeled ASO-CoA. Hydroxyapatite-purified proteins (20 µg) were incubated with 1 µCi of ^{125}I-labeled ASO-CoA in the presence (+) or in the absence (−) of 100 µm arachidonyl-CoA (A-CoA). After incubation at 0°C (10 min) and 37°C (3 min) the solution (50 µL) was UV-irradiated for 1 min. The proteins were separated on an SDS-polyacrylamide gel (12.5% T; 5% C). The gel was Coomassie-stained after electrophoresis (left); the distribution of radioactivity was monitored by autoradiography (right).

staining of the gel, the distribution of the radioactivity was monitored by autoradiography (**Fig. 3**).

As is clearly shown, only a few of the Coomassie-stained proteins could be detected by autoradiography (marked with *). The protein-bound radioactivity was reduced in the presence of arachidonyl-CoA. This result indicates that ASO-CoA is suited for the specific detection of acyl-CoA binding proteins.

DISCUSSION

Several attempts have been made in the past to characterize enzymes and proteins involved in membrane lipid biosynthesis using photoaffinity fatty acyl-CoAs as substrate analogues. Most of these studies have been carried out with a photolabel based on medium-chain ($C_{11–12}$) saturated fatty acids (24–30, 41–44); fewer studies were done with substances based on long-chain ($C_{16–18}$), saturated (45, 46) or unsaturated (41–43, 47) fatty acids, where some reactive groups are positioned within the hydrophobic chain, which may affect the binding of the analogue. Several proteins involved in lipid biosynthetic pathways have been examined with these analogues, e.g., acyl-CoA:cholesterol acyltransferase and cholesterol esterase (24), *N*-acylphosphatidylethanolamine synthase (29), acyl-CoA binding protein (25, 28), fatty acid binding protein (26, 45), acyl-CoA oxidase (27, 41), LAT (42, 43), or monoacylglycerol:acyl-CoA acyltransferase and diacylglycerol:acyl-CoA acyltransferase (30), but only few attempts have been made to characterize proteins specifically utilizing unsaturated fatty acids or acyl-CoAs.

Here we describe the synthesis and biological significance of a new photoaffinity, iodinable analogue of a physiological unsaturated fatty acyl-CoA, or, more exactly, of an oleic acid that was ω-terminally functionalized via an amino group. This fatty acid was coupled with a photoaffinity marker (azidosalicylic acid) and activated by converting it to its coenzyme A derivative to obtain ASO-CoA. This substance was used to characterize lysophosphatidylcholine:acyl-CoA-O-acyltransferase (LAT). Neither ASO-CoA nor the enzyme are commercially available.

LAT was shown to be stable against UV irradiation (data not shown), which may indicate that one possible function of the enzyme is to protect membrane lipids against UV damage. ASO-CoA is not recognized as a substrate for LAT, but in the dark it acts as a reversible competitor of arachidonyl-CoA.

This inhibition could not be explained by any non-specific detergent effect of this amphiphilic substance. Comparing the inhibitory potential of ASO-CoA with other detergent-like substances, it is clearly shown that the specific structure of a fatty acid is needed to inhibit the enzyme activity effectively. Even the pre-irradiated ASO-CoA was much less effective, indicating that the inihibition of the enzyme activity by ASO-CoA is not due to an overall substance characteristic (hydrophobicity), but to structure specificity, which was disrupted by irradiation. The fact that oleoyl-CoA acted as the best inhibitor of enzyme activity might indicate that both the unsaturated fatty acid and

coenzyme A are recognized by specific binding sites that increase the binding capacity of the enzyme.

After UV exposure, ASO-CoA inactivates the enzyme irreversibly, but this inactivation can be prevented when the exposure is done in the presence of arachidonyl-CoA. Arachidonyl-CoA is capable of protecting the enzyme against photo cross-linking and subsequent loss of activity by ASO-CoA. Thus it is concluded that ASO-CoA binds specifically to or nearby the catalytic site of the enzyme and is covalently photo cross-linked via its azido group with the enzyme.

Iodination of ASO-CoA at the 5′-position of the phenyl group offered the opportunity to use the label as a tool to visualize and analyze enzymes that utilize unsaturated fatty acids with a narrow range of specificity concerning the hydrophobic chain of the acid. Therefore, we used the iodinated ASO-CoA to identify some candidate proteins for LAT in a hydroxyapatite-purified fraction. It could be clearly shown that only some of the proteins within this protein source were capable of binding ASO-CoA specifically. Non-specific hydrophobic binding of ASO-CoA, which would not be reduced by arachidonyl-CoA, could be detected only for proteins with high molecular weight, indicating the existence of complexes containing proteins and lipids that bind fatty acids in a non-specific manner. These results indicate that ASO-CoA is suitable for identification of LAT or other acyl-CoA binding proteins. We are currently trying to visualize the LAT protein in more highly purified enzyme fractions by iodinated ASO-CoA, with the aim of microsequencing putative labeled proteins seen in SDS-gel electrophoresis.

Instead of iodination, the substance might also be coupled with biotin via the hydroxyl group of ASO-CoA to prevent handling with radioactive substances.

Furthermore, the above mentioned “model core substance” ω-amino-oleic acid might be useful in membrane lipid research and enzyme purification, e.g., the fatty acid or the corresponding acyl-CoA could be coupled with some chromatography matrices directly via its amino group, allowing the purification of enzymes that use fatty acid or acyl-CoA. In addition, ω-amino-oleic acid might also be a new and effective reagent for the chemical synthesis of photoaffinity lipids (42, 48) thereby acting as an additional tool for biomembrane research.

The described synthesis scheme (Scheme 1) could also be easily used to compose other amino-terminal functionalized unsaturated fatty acids with distinct positions of the double bond and corresponding derivatives by varying the agents to start with. For example, when using 1,7-heptandiole instead of 1,9-nonandiole for generating the analogue of component **1**, an analogue of palmitoleic acid would be obtained. For that reason this reaction scheme provides many possibilities for the generation of new tools for specific membrane lipid and protein research.

L. G. was supported by a doctoral fellowship from the Friedrich-Naumann-Stiftung, Königswinter, Germany. K. H. was supported by a doctoral fellowship from the Yamanouchi Research Institute, Oxford, UK. The excellent technical assistance of Christa Urban, Heike Hartmann-Ruff, and Annette Garbe is appreciated.

Manuscript received 19 May 1997, in revised form 10 November 1997, and in re-revised form 15 January 1998.

REFERENCES

1. Schultz, A. M., L. E. Henderson, and S. Oroszlan. 1988. Fatty acylation of proteins. *Annu. Rev. Cell. Biol.* **4:** 611–647.
2. Pfanner, N., L. Orci, B. S. Glick, M. Amherdt, S. R. Aeden, V. Malhotra, and J. E. Rothman. 1989. Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae. *Cell.* **59:** 95–102.
3. Lands, W. E., and G. C. Crawford. 1976. Enzymes of membrane phospholipid metabolism in animals. *In* The Enzymes of Biological Membranes. Vol. II. 1st ed. A. Martonosi, editor. Plenum Press, New York. 3–85.
4. Webster, G. R., and R. J. Alpern. 1964. Studies on the acylation of lysolecithin by rat brain. *Biochem. J.* **50:** 35–42.
5. Sastry, P. S., and L. E. Hokin. 1966. Studies on the role of phospholipids in phagocytosis. *J. Biol. Chem.* **241:** 3354–3361.
6. Stein, Y., C. Widnell, and O. Stein. 1968. Acylation of lysophosphatides by plasma membrane fractions of rat liver. *J. Cell. Biol.* **39:** 185–192.
7. Lands, W. E., M. Inoue, Y. Sugiura, and H. Okuyama. 1982. Selective incorporation of polyunsaturated fatty acids into phosphatidylcholine by rat liver microsomes. *J. Biol. Chem.* **257:** 14968–14972.
8. Irvine, R. F. 1982. How is the level of free arachidonic acid controlled in mammalian cells? *Biochem. J.* **204:** 3–16.
9. Pfannkuche, H. J., V. Kaever, D. Gemsa, and K. Resch. 1989. Regulation of prostaglandin synthesis by protein kinase C in mouse peritoneal macrophages. *Biochem. J.* **260:** 471–478.
10. Rode, H. N., M. Szamel, S. Schneider, and K. Resch. 1982. Phospholipid metabolism of stimulated lymphocytes. Preferential incorporation of polyunsaturated fatty acids into microsomal phospholipid upon activation with concanavalin A. *Biochim. Biophys. Acta.* **688:** 66–74.
11. Szamel, M., P. Berger, and K. Resch. 1986. Inhibition of T-lymphocyte activation by cyclosporin A: interference with the early activation of plasma membrane phospholipid metabolism. *J. Immunol.* **136:** 264–269.
12. Goppelt-Strübe, M., and K. Resch. 1987. Polyunsaturated fatty acids are enriched in plasma membranes of mitogen-stimulated T-lymphocytes. *Biochim. Biophys. Acta.* **904:** 22–28.
13. Szamel, M., B. Rehermann, B. Krebs, R. Kurrle, and K. Resch. 1989. Activation signals in human lymphocytes. Incorporation of polyunsaturated fatty acids into plasma membrane phospholipids regulates IL-2 synthesis via sustained activation of protein kinase C. *J. Immunol.* **143:** 2806–2813.
14. Szamel, M., F. Bartels, and K. Resch. 1993. Cyclosporin A inhibits T cell receptor-induced interleukin-2 synthesis of human T lymphocytes by selectively preventing a transmembrane signal transduction pathway leading to sustained activation of a protein kinase C isoenzyme, protein kinase C-beta. *Eur. J. Immunol.* **23:** 3072–3081.
15. Szamel, M., and K. Resch. 1995. T-Cell antigen receptor-induced signal transduction pathways. Activation and function of protein kinases C in T lymphocytes. *Eur. J. Biochem.* **228:** 1–15.
16. Mukherjee, A. B., L. Miele, and N. Pattabiraman. 1994. Phospholipase A_2 enzymes: regulation and physiological role. *Biochem. Pharmacol.* **48:** 1–10.
17. Lands, W. E., and P. Hart. 1965. Metabolism of glycerolipids. VI. Specificities of acyl-CoA: phospholipid acyltransferases. *J. Biol. Chem.* **240:** 1905–1911.
18. Reitz, R. C., M. El-Sheikh, W. M. Lands, I. Ismail, and F. D. Gunstone. 1969. Effects of ethylenic bond position upon acyltransferase activity with isomeric *cis*-octadecenoyl coenzyme A thiol esters. *Biochim. Biophys. Acta.* **176:** 480–490.
19. Sanford, G. L., and M. F. Frosolono. 1983. The role of acyl transferase in the biosynthesis of pulmonary microsomal phosphatidylglycerol. *Biochem. Biophys. Res. Commun.* **116:** 23–29.
20. Gavino, V. C., and D. W. Deamer. 1983. Lysophosphatidylcholine acyltransferase: purification and applications in membrane studies. *Ann. NY Acad. Sci.* **414:** 90–96.

21. Fyrst, H., D. V. Pham, B. H. Lubin, and F. A. Kuypers. 1996. Formation of vesicles by the action of acyl-CoA:1-acyllysophosphatidylcholine acyltransferase from rat liver microsomes: optimal solubilization conditions and analysis of lipid composition and enzyme activity. *Biochemistry.* **35:** 2644–2650.
22. Sanjanwala, M., G. Y. Sun, M. A. Cutrera, and R. A. MacQuarrie. 1988. Acylation of lysophosphatidylcholine in bovine heart muscle microsomes: purification and kinetic properties of acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine O-acyltransferase. *Arch. Biochem. Biophys.* **265:** 476–483.
23. Sanjanwala, M., G. Y. Sun, and R. A. MacQuarrie. 1989. Purification and kinetic properties of lysophosphatidylinositol acyltransferase from bovine heart muscle microsomes and comparison with lysophosphatidylcholine acyltransferase. *Arch. Biochem. Biophys.* **271:** 407–413.
24. Kinnunen, P. M., F. H. Klopf, C. A. Bastiani, C. M. Gelfman, and L. G. Lange. 1990. 12-[(5-Iodo-4-azido-2-hydroxybenzoyl)amino]dodecanoic acid: biological recognition by cholesterol esterase and acyl-CoA:cholesterol O-acyltransferase. *Biochemistry.* **29:** 1648–1654.
25. Hach, M., S. N. Pedersen, T. Borchers, P. Hojrup, and J. Knudsen. 1990. Determination by photoaffinity labelling of the hydrophobic part of the binding site for acyl-CoA esters on acyl-CoA-binding protein from bovine liver. *Biochem. J.* **271:** 231–236.
26. Gerber, G. E., D. Mangroo, and B. L. Trigatti. 1993. Identification of high affinity membrane-bound fatty acid binding proteins using a photoreactive fatty acid. *Mol. Cell. Biochem.* **123:** 39–44.
27. Mangroo, D., L. Steele, R. A. Rachubinski, and G. E. Gerber. 1993. Specific labeling of *Candida tropicalis* peroxisomal proteins with photoreactive fatty acid derivatives. *Biochim. Biophys. Acta.* **1168:** 280–284.
28. Atlasovich, F., J. A. Santome, and H. N. Fernandez. 1993. Photoreactive fatty acid analogues that bind to the rat liver fatty acid binding protein: 11-(5′-azidosalicylamido)-undecanoic acid derivatives. *Mol. Cell. Biochem.* **120:** 15–23.
29. McAndrew, R. S., B. P. Leonard, and K. D. Chapman. 1995. Photoaffinity labeling of cottonseed microsomal N-acylphosphatidylethanolamine synthase protein with a substrate analogue, 12-[(4-azidosalicyl)amino]dodecanoic acid. *Biochim. Biophys. Acta.* **1256:** 310–318.
30. Hare, J. F. 1995. Photolabeling acyl CoA binding proteins in microsome preparations. *FEBS Lett.* **375:** 188–192.
31. Kerkhoff, C., L. Gehring, K. Habben, K. Resch, and V. Kaever. 1996. Identification of two different lysophosphatidylcholine:acyl-CoA acyltransferases (LAT) in pig spleen with putative distinct topological localization. *Biochim. Biophys. Acta.* **1302:** 249–256.
32. Butenand, A., E. Hecker, M. Hopp, and W. Koch. 1962. Ueber den Sexuallockstoff des Seidenspinners. IV. Die Synthese des Bombykols und der *cis*/*trans*-isomeren Hexadecadien-(10.12)ole (1). *Liebigs Ann. Chem.* **658**: 39–64.
33. Goto, G., K. Okamoto, T. Okutani, and I. Imada. 1985. A facile synthesis of 1,4-benzochinones having a hydroxylalkyl side chain. *Chem. Pharm. Bull.* **33**: 4422–4431.
34. Goto, G., T. Shima, H. Masuya, Y. Masuoka, and K. Hiraga. 1975. Stereoselective synthesis of (2;E)-9,11-tetradecadienyl-1-acetate, a major component of the sex pheromone of *Spodoptera litura*. *Chem. Lett.* **1975:** 103–106.
35. Bestmann, H. J., W. Stransky, and O. Vostrowsky. 1976. Darstellung lithiumsalzfreier Ylidlösungen mit Natrium-bis(trimethylsilyl)amid als Base. *Chem. Ber.* **109:** 1694–1700.
36. Poleschner, H., M. Heydenreich, and D. Martin. 1991. Cyclische Ether als Edukte zur Synthese von Schmetterlings-Pheromonen. *Synthesis.* **1991:** 1231–1235.
37. Bishop, J. E., and A. K. Hajra. 1980. A method for the chemical synthesis of ^{14}C-labeled fatty acyl coenzyme A's of high specific activity. *Anal. Biochem.* **106:** 344–350.
38. Cailla, H. L., C. J. Vannier, and M. A. Delaage. 1976. Guanosine 3′,5′-cyclicmonophosphate assay at 10^{-15}-mole level. *Anal. Biochem.* **70:** 195–202.
39. Wessel, D., and U. I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138:** 141–143.
40. Nakamura, K., and S. Handa. 1984. Coomassie brillant blue staining of lipids on thin-layer plates. *Anal. Biochem.* **142:** 406–10.
41. Rajasekharan, R., R. C. Marians, J. M. Shockey, and J. D. Kemp. 1993. Photoaffinity labeling of acyl-CoA oxidase with 12-azidooleoyl-CoA and 12-[(4-azidosalicyl)amino]dodecanoyl-CoA. *Biochemistry.* **32:** 12386–12391.
42. Rajasekharan, R., and V. Nachiappan. 1994. Use of photoreactive substrates for characterization of lysophosphatidate acyltransferases from developing soybean cotyledons. *Arch. Biochem. Biophys.* **311:** 389–394.
43. Rajasekharan, R., V. Nachiappan, and H. S. Roychowdhury. 1994. Photolabeling of soybean microsomal membrane proteins with photoreactive acyl-CoA analogs. *Eur. J. Biochem.* **220:** 1013–1018.
44. Jezek, P., J. Hanus, C. Semrad, and K. D. Garlid. 1996. Photoactivated azido fatty acid irreversibly inhibits anion and proton transport through the mitochondrial incoupling protein. *J. Biol. Chem.* **271:** 6199–6205.
45. Waggoner, D. W., J. A. Manning, N. M. Bass, and D. A. Bernlohr. 1991. In situ binding of fatty acids to the liver fatty acid binding protein: analysis using 3-[^{125}I]iodo-4-azido-N-hexadecylsalicylamide. *Biochem. Biophys. Res. Commun.* **180:** 407–415.
46. Woldegiorgis, G., J. Lawrence, A. Ruoho, T. Duff, and E. Shrago. 1995. Photoaffinity labeling of mitochondrial proteins with 2-azido [^{32}P]palmitoyl CoA. *FEBS Lett.* **364:** 143–146.
47. Rossi, J. P., J. M. Delfino, A. J. Caride, and H. N. Fernandez. 1995. Interaction of unsaturated fatty acids with the red blood cell Ca^{2+}-ATPase. Studies with a novel photoactivatable probe. *Biochemistry.* **34:** 3802–3812.
48. Van der Bend, R. L., J. Brunner, K. Jalink, E. J. Van Corven, W. H. Moolenaar, and W. J. van Blitterswijk. 1992. Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO J.* **11:** 2495–2501.