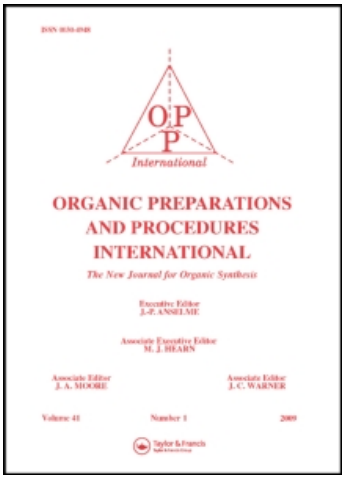

Organic Preparations and Procedures International
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t902189982

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[a] Department of Chemistry, New Mexico State University, Las Cruces, NM [b] Laboratory of Biological Chemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD

To cite this Article Pankayatselvan, Ratna , Guziec Jr., Frank S. , Gopalan, Aravamudan S. , Raghavachari, Ramesh , Guziec, Lynn James , Wei, Dongchu , Felsted, Ronald L. and Glover, Constance J.(1995) 'THE PREPARATION OF SOME ANALOGUES OF MYRISTOYL CoA', Organic Preparations and Procedures International, 27: 3, 347 — 354
To link to this Article: DOI: 10.1080/00304949509458467
URL: http://dx.doi.org/10.1080/00304949509458467

ORGANIC PREPARATIONS AND PROCEDURES INT., 27 (3), 347-354 (1995)

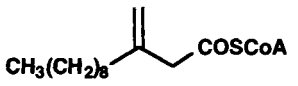
THE PREPARATION OF SOME ANALOGUES OF MYRISTOYL CoA

Ratna Pankayatselvan*†, Frank S. Guziec, Jr.†, Aravamudan S. Gopalan†, Ramesh Raghavachari†, Lynn James Guziec†, Dongchu Wei†, Ronald L. Felsted†† and Constance J. Glover††

†*Department of Chemistry, New Mexico State University, Las Cruces, NM 88003-0001*

††*Laboratory of Biological Chemistry, National Cancer Institute, National Institutes of Health, Building 37, Room 5DO2, Bethesda, MD 20892*

Acylation of cellular and viral proteins by fatty acids has recently been recognized as an important post-translational protein modification. In some cases such protein acylation may be necessary for viral replication and retroviral induced events such as tumorigenesis and immunosuppression. N-Myristoylation of the N-terminal glycine of various cellular, oncogenic and viral proteins appears to be a particularly important modification and is the subject of a number of recent reviews.[1-3] The design of specific inhibitors of myristoylation as therapeutic agents to interfere with cellular transformation or viral replication is currently an important target in drug development.[3]



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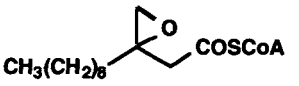

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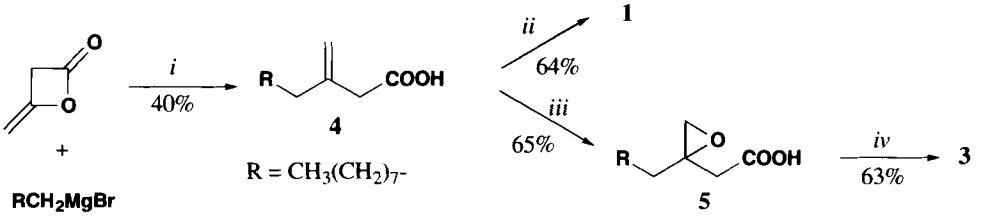
Myristoylation of the protein N-terminal glycine is characterized by a very high specificity for myristoyl CoA as the acyl donor. One possible selective target for blocking N-myristoylation is the enzyme which transfers myristate from myristoyl CoA to the N-terminal glycine of peptide substrates, namely myristoyl CoA: N-myristoyltransferase (NMT).[3] In the design of inhibitors for this enzyme, we were interested in preparing structural analogues of myristoyl CoA that could function as irreversible inhibitors of NMT by covalent bond formation at the active site of the enzyme. Also considered were non-hydrolyzable fatty acid S-alkylated CoA analogues that could exhibit preferred binding to the enzyme but which could not participate in the acyl transfer process. This paper describes the details of the preparation and characterization of some examples of these new types of inhibitors of NMT.

Our synthetic targets **1-3** incorporated modified fatty acid moieties appended to CoA groups to facilitate binding to NMT. In the first two cases, acyl CoA derivatives containing reactive β,γ-unsaturated and β,γ-epoxy fatty acyl groups were chosen. These groups could potentially alkylate at the active site of the tranferase upon binding, leading to irreversible inhibition of the enzyme. A third

target introduced a non-hydrolyzable S-Alkyl CoA linkage onto a N-myristoyl glycinamide derivative. The incorporation of fatty acid, amino acid and CoA moieties into one substrate was expected to increase its binding to NMT leading to competitive inhibition of the enzyme. While this work was in progress, the design and synthesis of another inhibitor of NMT, S-(2-oxopentadecyl)-CoA using a non-hydrolyzable S-CoA linkage was reported.[4]

Our synthetic strategy, first involved the development of methods for the preparation of the selected modified fatty acid precursors, which could then be coupled to coenzyme A. As depicted in **Scheme 1**, the key intermediate for the synthesis of coenzyme A derivatives **1** and **2** was the 3-methylenedodecanoic acid (**4**). This acid was prepared in moderate yields by the reaction of diketene with



i) CoI_2, -78°, THF; *ii*) a) CH_2Cl_2, $(COCl)_2$ or ClCOOEt, EtN*i*-Pr_2 b) CoASNa, BHT,THF/H_2O; *iii*) *m*-CPBA, CH_2Cl_2 *iv*) a) ClCOOEt, EtN*i*-Pr_2 b) CoASNa, BHT,THF/H_2O

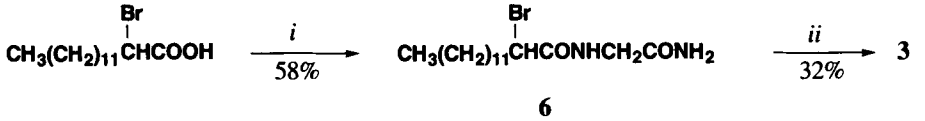
Scheme 1

nonylmagnesium bromide in the presence of cobalt (II) iodide at -78° in THF.[5] Two alternative procedures[6,7] then were successfully utilized for the coupling of acid **4** with coenzyme A. Treatment of **4** with excess oxalyl chloride in methylene chloride gave the corresponding acid chloride which was coupled to the sodium salt of coenzyme A in aqueous THF solution buffered with 150 mM sodium bicarbonate (pH 8.8), following published procedures.[6] The second method involved the condensation of **4** with ethyl chloroformate and subsequent reaction of the mixed anhydride intermediate with coenzyme A sodium salt, once again in buffered aqueous THF solution.[7] Both routes gave the coenzyme A derivative **1** in about 60% yield after purification by precipitation with 1.3% perchloric acid and washing with acetone and diethyl ether.

Epoxidation of **4** with m-chloroperbenzoic acid proceeded slowly but cleanly to give **5**. The epoxy acid **5** was unstable upon storage and exposure to column chromatographic conditions, but could be used directly in the CoA coupling reactions. Attempts to prepare the acid chloride of **5** with oxalyl chloride was accompanied by substantial destruction of the epoxide moiety. However, the mixed anhydride could be prepared by the condensation of **5** with ethyl chloroformate in the presence of N,N-diisopropylethylamine. After purification of the mixed anhydride by extraction into pentane, it was successfully coupled to the sodium salt of coenzyme A. The resulting coenzyme A derivative **2** was prepared and purified by a procedure analogous to that already described for the synthesis of **1**.

For the preparation of S-alkylated coenzyme A derivative **3**, 2-bromotetradecanoyl chloride

was coupled with glycinamide in methylene chloride at 0° in the presence of N,N-diisopropylethylamine. The resulting amide **6**, was isolated in 58% yield, as colorless crystals after recrystallization from absolute ethanol. Alkylation of coenzyme A with **6** was carried out in aqueous THF solution buffered to pH 8.8 with 150 mM sodium bicarbonate, in the absence of oxygen, and proceeded slowly



i) a) $(COCl)_2$, C_6H_6, DMF (cat.), b) Glycinamide, EtN*i*-Pr_2, CH_2Cl_2, *ii*) CoASNa, THF/H_2O

Scheme 2

at room temperature. The progress of the reaction was followed spectrophotometrically by monitoring the disappearance of the free thiol group of coenzyme A, using DTNB reagent.[8] The crude product, having some residual coenzyme A, was purified by preparative reverse phase thin layer chromatography and **3** was isolated as a colorless solid, homogeneous on TLC. The methodology described above provides ready access to the target compounds and is amenable for the preparation of other analogs. It should be noted that while S-alkylation of coenzyme A with alkyl halides[9] and α-haloketones[4,10] are known, the corresponding alkylation with 2-haloamides has not been reported. The compounds that were synthesized were then assayed for their inhibitory activity. Under the assay conditions, none of the compounds described above were found to irreversibly inhibit NMT. The details of the purification of NMT and the results of the biological assays have been reported elsewhere.[11]

In conclusion, methodology for the preparation of some functionalized fatty acid coenzyme A derivatives has been successfully developed. These classes of compounds are of growing interest for their potential to function as irreversible inhibitors of NMT. The design of improved inhibitors of NMT based on our initial studies and also other approaches to its inhibition are currently under investigation.

EXPERIMENTAL SECTION

^{1}H-NMR data were obtained at 200 MHz on a Varian XL-200 spectrometer in $CDCl_3$ or D_2O solvents as specified in the text using TMS or DSS as internal reference. Infrared spectra were recorded on a Perkin Elmer 1600 series FTIR. UV spectra were obtained using a Hewlett Packard 8452A Diode Array Spectrophotometer. Elemental analysis were performed by Galbraith Laboratories, Knoxville, TN and Desert Analytics, Tuscon, AZ. Reverse phase Si-C_{18}F TLC plates (Baker Cat # 7013-00) and Cellulose plates (Kodak 13254) were used for the analysis of the CoA derivatives. All solvents were dried and distilled by standard procedures before use.

3-Methylenedodecanoic Acid (4).- In a three necked flask fitted with a reflux condenser, dry magnesium turnings (0.70 g, 28.8 mmol) were suspended in freshly distilled THF (10 mL). Approximately 1g of 1-bromononane was pipetted to the surface of the magnesium and the solution was warmed.

Once a vigorous reaction was observed, the remaining bromononane (in all 5.0 g, 24 mmol) was added dropwise in 10 mL of THF. The resulting Grignard reagent was stirred overnight under nitrogen atmosphere to ensure its complete formation.

To a mixture of CoI_2 (0.75 g, 2.4 mmol) and freshly distilled THF (50mL) in a three necked flask at -78° in a dry ice/acetone bath, diketene (1.9 g, 22.6 mmol) was added. The Grignard reagent prepared as described above, was added dropwise into the cooled solution. The color changed from dark green to a brown-green. The mixture was stirred at -78° for 6 hrs. The reaction was carefully neutralized to pH 7 with 6 M HCl; it was then extracted with ether and the ethereal layer was washed with water and brine. The combined ether layer was dried over $MgSO_4$. Removal of the solvent resulted in a brownish yellow solid (2.94 g, 60% yield). Kugelrohr distillation (95°/0.1 torr) gave an oil which was recrystallized from ethanol to afford white crystals, mp 40°, analytically pure (1.95 g, yield 40%). ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.27 (m, 14H), 2.12 (t, 2H), 3.09 (s, 2H), 3.95(d, 2H). IR ($CDCl_3$): 1645, 1710, 2930 cm^{-1}.

Anal. Calcd for $C_{13}H_{25}O_2$: C, 73.19; H, 11.81. Found: C, 73.53; H, 11.74

3-(Epoxymethylene)dodecanoic Acid (5).- To 3-methylenedodecanoic acid (4) (0.20 g, 0.94 mmol) in methylene chloride (5 mL), m-chloroperbenzoic acid (0.406 g, 1.88 mmol) in methylene chloride (5 mL) was added dropwise. The reaction was allowed to stir at room temperature in the dark for 48 hrs and then was poured into 10% sodium sulfite solution and stirred for 30 minutes. The organic layer was separated and washed with water, brine and dried over sodium sulfate. Removal of the solvent resulted in a viscous colorless liquid (220 mg, crude yield 65%). The product was unstable to column chromatography and Kugelrohr distillation. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.26 (m, 14H), 1.70 (m, 2H), 2.70 (q, 2H), 2.76 (s, 2H). IR (neat): 1710, 2920 cm^{-1}. Stability tests: 30 mg of the epoxide was placed in $CDCl_3$. One sample was stored at room temperature and another kept at -12°. NMR spectra of the sample was taken periodically. After 8 days the sample kept at room temperature began to show decomposition. Even after 24 days, the freezer sample showed only slight decomposition.

3-Methylenedodecanoyl CoA (1) [*via* ***the acid chloride method***].- To 3-methylene-dodecanoic acid (**4**) (250 mg, 1.17mmol) in methylene chloride (12 mL), excess oxalyl chloride (1.50 g, 11.7 mmol) was added and the mixture stirred for 16 hours under nitrogen atmosphere. The solvent and the excess oxalyl chloride was removed under reduced pressure, and the acid chloride directly used in the next step. NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.27 (m, 14H), 2.10 (t, 2H), 3.56 (s, 2H), 5.03 (d, J = 12Hz, 2H). IR (neat): 1819, 1760 cm^{-1}.

To a mixture of acid chloride (289 mg, 1.26 mmol) and BHT (278 mg, 1.26 mmol), 26 mL of a solution of CoASNa (311 mg, 39.2 mmol) in freshly distilled tetrahydrofuran-aqueous 150 mM sodium bicarbonate buffer (2.2: 1, pH 8.8) was added. The reaction mixture was incubated for 30 minutes at 37°, and the pH was maintained at 8.5 by the addition of dilute sodium hydroxide during this period. The pH was then adjusted to 4.5 by careful addition of 10% perchloric acid (0.25 mL). Then the THF was completely removed by bubbling nitrogen followed by rotary evaporation. The residual aqueous solution was lyophilized overnight to get a white fluffy powder (810 mg). To this

solid 75 mL of chilled 1.3% perchloric acid was added, the mixture vortexed and centrifuged at 18,000 rpm at 4° for fifteen minutes. The filtrate was decanted and the solid residue was again washed with 75 mL of 1.3% perchloric acid in a similar manner. The solid obtained was then washed with dry acetone (30 mL), followed by twice with ether (2 x 30 mL). After drying under reduced pressure, **1** was obtained as a white fluffy solid (242 mg, 64%). UV (H_20): λ max 258; $A_{232}/A_{260} = 0.50$

The concentration and purity of the acyl CoA's are determined spectrophotometrically by measuring the ratio of the absorbances at 232 nm and 260 nm. The Coenzyme A shows A_{232}/A_{260} ratio of 0.25, while the acyl CoA derivatives have A_{232}/A_{260} ratio of 0.50 - 0.56. TLC: R_f = 0.77 (n-BuOH: H_2O: AcOH = 5: 3: 2) on cellulose plate.

Anal. Calcd for $C_{34}H_{58}N_7O_{17}P_3S{\bullet}H_2O$: C, 41.67; H, 6.17; N, 10.01. Found: C, 41.86; H, 6.20; N, 9.97

3-Methylenedodecanoyl CoA (1) [*via the mixed anhydride*].- To 3-methylenedodecanoic acid (**4**) (100 mg, 0.47mmol) in methylene chloride (5 mL), diisopropylethylamine (60 mg, 0.47 mmol) and ethyl chloroformate (200 mg, 1.9 mmol) was added and the mixture stirred for two hours. The solvent and the excess reagent was removed under reduced pressure to give the anhydride as a near colorless oil. The crude anhydride was used directly for reaction with the sodium salt of CoA. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.27 (m, 14H), 1.37 (t, 3H), 2.13 (t, 2H), 3.17 (s, 2H), 4.33 (q, 2H), 5.02 (d, 2H). IR (neat): 1819, 1760 cm^{-1}.

To the mixture of the acid anhydride (185 mg) and BHT (103 mg, 0.47 mmol), 13 mL of a solution of CoASNa (115 mg, 14.5 mmol) in freshly distilled tetrahydrofuran-aqueous 150 mM sodium bicarbonate buffer (2.2: 1, pH=8.8) was added. The reaction mixture was incubated for 100 minutes at 37°. No change in pH was observed during the reaction. The disappearance of the thiol group was monitored by adding 5 mL aliquot of the reaction mixture to 5 mL of 0.1 mM DTNB[8] solution adjusted to pH 8.5 with 50 mM of Tris buffer. The absorbance at 412 nm was read against a 0.1 mM DTNB as a blank. The absorbance reading immediately after mixing was 0.9; and the reaction was allowed to progress until a constant absorbance was observed (0.25).

The pH was then adjusted to 4.5 by careful addition of 10% perchloric acid (0.25 mL). Then the THF was completely removed by bubbling nitrogen followed by rotary evaporation. The residual aqueous solution was lyophilized overnight to get a white fluffy powder (330 mg). To this solid 25 mL of chilled 1.3% perchloric acid was added, the mixture vortexed and centrifuged at 18,000 rpm at 4° for fifteen minutes. The filtrate was decanted and the solid residue was again washed with 25 mL of 1.3% perchloric acid in a similar manner. The solid obtained was then washed with dry acetone (10 mL), followed by twice with ether (2 x 10 mL). After drying *in vacuo*, **1** was obtained as a white solid (85 mg, 61% yield). The spectral, UV and TLC data were identical with that of the CoA derivative prepared by the acid chloride method.

3-(Epoxymethylene)dodecanoyl CoA (2).- To 3-(epoxymethylene)dodecanoic acid (**5**) (100 mg, 0.44 mmol) in methylene chloride (5 mL), diisopropylethylamine (56 mg, 0.44 mmol) and ethyl chloroformate (238 mg, 2.2 mmol) was added and the mixture stirred for 2 hrs. Then the solvent was removed under reduced pressure and the residue was washed with pentane twice. The pentane wash-

ings were combined and the solvent removed to give a colorless oil (60 mg).^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.26 (m, 14H), 1.37 (t, 3H), 1.56 (m, 2H), 2.75 (s, 2H), 2.55, 2.95 (dd, J = 15Hz, 2H), 4.34 (q, J = 7 Hz, 2H). IR (neat): 1819, 1766 cm^{-1}.

To the mixture of the mixed anhydride (60 mg) and BHT (103 mg, 0.47 mmol), 13 mL of a solution of CoASNa (115 mg, 14.5 mmol) in freshly distilled tetrahydrofuran-aqueous 150 mM sodium bicarbonate buffer (2.2: 1, pH 8.8) was added. The reaction mixture was incubated for 100 minutes at 37°. No change in pH was observed during the reaction. The disappearance of the thiol group was monitored by adding a 2 mL aliquot of the reaction mixture to 2 mL of 0.1 mM DTNB[8] solution adjusted to pH 8.5 with 50 mM of Tris buffer. The absorbance at 412 nm was read against a 0.1 mM DTNB solution as a blank.

The pH was then adjusted to 4.5 by careful addition of 10% perchloric acid (0.25 mL). Then the THF was completely removed by bubbling nitrogen followed by rotary evaporation. The residual aqueous solution was lyophilized overnight to get a white fluffy powder (232 mg). To this solid 20 mL of chilled 1.3% perchloric acid was added, the mixture vortexed and centrifuged at 18,000 rpm at 4° for fifteen minutes. The filtrate was decanted and the solid residue was again washed with 20 mL of 1.3% perchloric acid in a similar manner. The solid obtained was then washed with dry acetone (10 mL), followed by twice with ether (2 x 10 mL). The product was dried under reduced pressure and **2** was obtained (89 mg, 63% yield). UV (H_2O): λ max 258; A_{232}/A_{260} = 0.5; TLC: R_f = 0.71 [*n*-BuOH: H_2O:AcOH = 5:3:2] on a cellulose plate.

Anal. Calcd. for $C_{34}H_{58}N_7O_{18}P_3S \cdot 2H_2O$: N, 9.67; P, 9.17; S, 3.16. Found: N, 9.49; P, 8.84; S, 3.21

N-(2-Bromotetradecanoyl)glycinamide (6).- Oxalyl chloride (0.62 g, 0.45 mL, 4.89 mmol) was added in one portion to a solution of 2-bromotetradecanoic acid (1.0 g, 3.26 mmol), in dry benzene (5 mL) containing a catalytic amount of N,N-dimethylformamide (0.024 mL, 0.32 mmol). The reaction mixture was stirrred at room temperature for 5 hrs. The solution was decanted from a small amount of gummy material and concentrated under reduced pressure at 40°. Bulb to bulb distillation at 8 torr afforded the 2-bromotetradecanoic acid chloride as a colorless liquid (1.0 g, 95% yield). ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.2-1.55 (bm, 22H), 4.12 (m, 2H), 5.40 (dd,1H, J = 8 Hz). IR (neat): 1780 cm^{-1}.

To a mixture of 2-bromotetradecanoyl chloride (5.09 g, 15.6 mmol), glycinamide (1.72 g, 15.55 mmol) and methylene chloride (50 mL) was added dropwise at 0° solution of N,N-diisopropylethylamine (5.4 mL, 31.1 mmol) in methylene chloride (25 mL). The mixture was stirred overnight at room temperature. Water (30 mL) was added and the resulting colorless crystals were washed with water. Recrystallization from absolute ethanol afforded **6** as colorless crystals (yield 58%), mp 142-144°. ^{1}H NMR ($CDCl_3$-DMSO-d_6): δ 0.87 (t, 3H, J =2 Hz)1.18-1.31 (bm, 22H), 1.91 (m, 3H), 3.36 (m, 2H), 4.47 (t, 1H, J = 7 Hz), 7.03 (bs, 1H), 7.28 (bs, 1H), 8.38 (bs, 1H). IR (KBr): 3350, 3170, 1675 and 1640 cm^{-1}.

Anal. Calcd for $C_{16}H_{31}BrN_2O_2$: C, 52.89; H, 8.60; N, 7.71. Found: C, 53.02; H, 8.86; N, 7.98

N-(2-SCoA tetradecanoyl)glycinamide Tetrasodium Salt (3).- To the sodium salt of CoA (50 mg; 0.065 mmol) and dithiothreitol (2 mg, 0.013 mmol) in 1 mL of freshly degassed and deionized water, sodium bicarbonate solution was added (0.15 mM solution, 3 mL) until pH was 8.8. A solution of the bromoglycinamide **6** (50 mg, 0.138 mmol) in freshly distilled THF (12 mL) was prepared. The CoA solution was added all at once to the solution of **6.** The reaction mixture was then degassed by bubbling nitrogen for five minutes immediately after mixing. The pH of the reaction mixture was 10. The disappearance of the free thiol group was monitored by DTNB[8] analysis. After 24 hrs the reaction mixture was poured into acetone (50 mL). The precipitate was centrifuged and the supernatent was removed. The precipitate was washed with acetone (2 x 12 mL) and then dried under reduced pressure to give 93 mg of a white solid. This solid was purified by chromatography on reverse phase tlc plates using 7: 3 methanol: water system and extracted with 70% methanol in water. After removal of methanol and subsequent lyophilization **3** was obtained as a pure white solid (24 mg, 32% yield). TLC analysis of the solid gave a single spot on a cellulose plate R_f = 0 (*n*-BuOH: AcOH: H_2O = 5:2:3), that did not correspond to coenzyme A.

Anal. Calcd for $C_{37}H_{62}N_9O_{18}P_3SNa_4 \cdot 12H_2O$: N, 9.30; P, 6.87; S, 2.36. Found: N, 9.45; P, 6.63; S, 2.11

Acknowledgement.- This investigation was supported by the National Cancer Institute, Contracts NO1- CM-87278 and NO1-CM-17528.

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(Received February 4, 1994; in revised form October 14, 1994)