Novel fatty acyl substrates for myristoyl-CoA:protein N-myristoyl-transferase

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Abstract Myristoyl-CoA:protein N-myristoyltransferase (NMT) catalyzes the covalent attachment of myristic acid to the NH_2-terminal Gly residues of a number of viral and cellular proteins. The remarkable specificity of this enzyme for myristoyl CoA observed in vivo appears to arise in large part from a cooperativity between NMT's acylCoA and peptide binding sites: the length of the acylCoA bound to NMT influences the interactions of peptide substrates with NMT. We have previously synthesized analogs of myristic acid with single oxygen or sulfur for methylene substitutions. These heteroatom substitutions produce significant reductions in acyl chain hydrophobicity without accompanying alterations in chain length or stereochemical restrictions. In vitro studies have shown that the CoA thioesters of these analogs are substrates for *S. cervisiae* NMT and that the efficiency of their transfer to octapeptide substrates is peptide sequence-dependent. In vivo studies with cultured mammalian cells have confirmed that these fatty acid analogs are selectively incorporated into a subset of cellular N-myristoylproteins, that only a subset of analog-substituted proteins undergo redistribution from membrane to cytosolic fractions, and that these analogs can inhibit the replication of human immunodeficiency virus I and Moloney murine leukemia viruses—two retroviruses that depend upon N-myristoylation of their gag polyprotein precursors for assembly. We have now extended our analyis of NMT-acylCoA interactions by synthesizing additional analogs of myristic acid and testing them in a coupled in vitro assay system. Myristic acid analogs with two oxygen or two sulfur substitutions have hydrophobicities comparable to that of hexanoic acid and decanoic acid, respectively. Despite this large reduction in hydrophobicity, they are utilized as substrates by *S. cerevisiae* NMT in vitro, albeit less efficiently than the single heteroatom-substituted compounds. The branched chain fatty acids isomyristic acid and anteisopentadecanoic acid are remarkably good NMT substrates. In addition, two aromatic ring-containing myristic acid analogs will serve as substrates for NMT. Introduction of a *cis* double bond between carbons 8 and 9 of the aromatic fatty acid analog 11-phenylundecanoic acid makes this compound a much better substrate for NMT. The data confirm that the acylCoA bound to NMT can dramatically influence the interaction of NMT with synthetic octapeptide substrates. These novel NMT substrates, which differ markedly in their physical-chemical properties from myristic acid, may represent useful compounds for investigating the role of fatty acylation in protein targeting and function.—**Heuckeroth, R. O., E. Jackson-Machelski, S. P. Adams, N. S. Kishore, M. Huhn, A. Katoh, T. Lu, G. W. Gokel, and J. I. Gordon.** Novel fatty acyl substrates for myristoyl-CoA:protein N-myristoyl-transferase. *J. Lipid Res.* 1990. **31:** 1121–1129.

Supplementary key words protein N-myristoylation • heteroatom-containing fatty acid analogs • myristoyl-CoA:protein N-myristoyl-transferase • fatty acid-protein interactions

N-myristoyl proteins contain a 14-carbon saturated fatty acid attached to their NH_2-terminal Gly residues via an amide linkage (reviewed in refs. 1–4). In vivo, N-myristoylproteins do not incorporate fatty acids of other chain lengths to any significant extent (6–14). Myristoyl-CoA:protein N-myristoyltransferase (NMT; E.C. 2.3.1.97) catalyzes this co-translational modification (15). *S. cerevisiae*, wheat germ, and rat liver NMTs all exhibit a high degree of acyl chain selectivity (16–18). We have used an in vitro assay for NMT (19) to examine the basis of its acyl CoA specificity (20). Analysis of naturally occurring acyl CoAs of varying chain length revealed an apparent cooperativity between the enzyme's acylCoA and peptide binding sites. In fact, much of NMT's acyl CoA specificity appears to occur through the influence of the bound acyl CoA on NMT-peptide interactions.

We have previously synthesized a series of heteroatom analogs of myristic acid to examine both the biology and enzymology of protein N-myristoylation (17, 20). Many of these compounds contained single oxygen or sulfur for methylene substitutions. Introduction of the heteroatom results in loss of a site for hydrophobic interaction and generation of a hydrogen bond acceptor. Thus, while these

Abbreviations: NMT, myristoyl-CoA:protein N-myristoyltransferase; BOC, t-butyloxycarbonyl; FMOC, fluorenylmethyloxycarbonyl; THF, tetrahydrofuran; HPLC, high performance liquid chromatography; HIV-I, human immunodeficiency virus-I; MoMLV, Moloney murine leukemia virus.

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analogs are predicted to be similar in length to myristic acid (21), their hydrophobicities are comparable to those of dodecanoic or decanoic acid (20, 22). In vitro studies (20) indicate that the CoA thioesters of many of these analogs bind to *S. cerevisiae* NMT with affinities comparable to that of myristic acid. However, the efficiency of their transfer to octapeptide substrates depends on both the peptide sequence and fatty acid analog structure. These observations may reflect subtle alterations in the peptide binding site produced by the interaction of analog CoA with NMT. The in vitro studies with heteroatom-substituted analogs also demonstrated that NMT selects its acyl CoA substrates primarily on the basis of chain length and not hydrophobicity (20).

The reason for selection of myristic acid for covalent attachment to viral and cellular proteins is largely unknown. Myristate is a relatively rare acyl chain, comprising ~1% of cellular fatty acids in mammalian cells (23). To investigate the role of the myristoyl moiety in N-myristoylproteins, we performed metabolic labeling studies with a tritium-labeled analog of myristic acid- 10- (propoxy)decanoic acid (11-oxamyristic acid[2]) and several cultured mammalian (rat and mouse) cell lines (24). This compound's hydrophobicity is comparable to that of decanoic acid (20). Only a subset of cellular N-myristoylproteins incorporated the analog. In addition, only a subset of analog-substituted proteins underwent redistribution from membrane to cytosolic fractions (24). These results demonstrated that the analog could enter cells, be converted to its CoA thioester by mammalian CoA ligase(s), and serve as an alternative substrate for a mammalian NMT. In addition, the in vitro assay, which used *S. cerevisiae* NMT, accurately predicted the selective incorporation observed in vivo by showing sequence-dependent incorporation of the analog into octapeptide substrates. Finally, these observations suggested that the hydrophobicity of the acyl chain is more important for the membrane association of some N-myristoyl proteins than of others, and that cellular NMT activity could be exploited to deliver heteroatom-containing analogs to specific N-myristoylproteins.

Site-directed mutagenesis of the Gly^1 residues of the $Pr65^{gag}$ of Moloney murine leukemia virus (MoMLV, ref. 26) and the $Pr55^{gag}$ of human immunodeficiency virus I (HIV-I, ref. 27) blocks their N-myristoylation and subsequent targeting to the plasma membrane where viral assembly occurs. We have recently demonstrated that replication of HIV-I and the MoMLV can be inhibited by heteroatom-containing analogs of myristic acid without accompanying cellular toxicity (28). Interestingly, the myristic acid analog that inhibits HIV-I replication does not affect MoMLV replication. This may reflect differences in the incorporation of particular analogs into specific N-myristoylprotiens or differences in the structural characteristics required for the function of particular acylproteins.

It is likely that the myristoyl groups of N-myristoylproteins play a structural role that extends beyond membrane association. Not all N-myristoylproteins are membrane bound. For example, the catalytic subunit of cAMP-dependent protein kinase (PK-A) behaves as a soluble protein once it is released from its regulatory subunit (29). The polyoma virus VP2 protein appears to be associated with the nucleoskeleton (30). High resolution X-ray crystallographic studies (31) and site-directed mutagenesis experiments (32) indicate that the myristic acid moiety of poliovirus' VP4 capsid protein is well ordered, involved in protein-protein interactions, and critical for viral capsid assembly. N-myristoylation, as a co-translational modification, may also influence protein folding. For these functions, the hydrophobicity of the acyl chain may be less important that its precise structure.

In this paper, we report the synthesis of additional analogs of myristic acid and show that NMT may be able to utilize a wider variety of fatty acyl CoA substrates than previously expected. These fatty acyl analogs not only provide new insights about NMT- ligand interactions, but may also provide new opportunities to investigate the role of the myristoyl moiety in acylprotein function.

EXPERIMENTAL PROCEDURES

Materials

Pseudomonas CoA ligase (acyl coenzyme A synthetase, E.C. 6.2.1.3) was purchased from Sigma (St. Louis, MO). NMT was partially purified (~150-fold) from *S. cerevisiae* strain BJ405 (33) by ammonium sulfate precipitation, and then DEAE Sepharose CL6B and agarose CoA chromatography (16). Isomyristic acid and anteisopentadecanoic acid were kindly supplied by David Silbert (Washington University).

Synthesis of a tritiated octapeptide substrate of NMT

The 9-fluorenylmethyloxycarbonyl derivative of L-alanine (FMOC-[^{3}H]-L-Ala) (6.5 mg, 1.5 Ci/mmol) was prepared by reacting L-alanine (6.5 mg, 200 mCi of [2,3-^{3}H]-L-alanine, 60 Ci/mmol) with FMOC-N-hydroxysuccinimide ester as described (34). The peptide intermediate Ala-Ser(benzyl)-Ala-Arg(*p*-toluenesulfonyl)- Arg(*p*-toluenesulfonyl)-resin was prepared using an Applied Biosystems Model 430A peptide synthesizer. T-butyloxy-

[2]In this and previous reports (17, 20, 24) we have used the nomenclature of Pascal, Mannarell, and Ziering (25) when referring to the heteroatom-substituted analogs of myristate. In this scheme, the type and position of the heteroatom is taken into consideration as is analog chain length.

carbonyl (BOC) amino acids were sequentially added to *p*-methylbenzhydrylamine resin (97 mg, 0.47 μmol NH_2/mg resin) using dicylcohexylcarbodiimide/N-hydroxybenzotriazole (DCC/HOBT) double coupling cycles as recommended by the manufacturer. FMOC-[^{3}H]-L-Ala (6.5 mg, 60 μmol) was coupled to the intermediate peptide-resin with DCC/HOBT, and a second coupling with unlabeled FMOC-L-Ala was performed to ensure that the reaction was complete. Following removal of the FMOC group with 10% piperidine/dichloromethane, the synthesis was completed by the sequential addition of BOC-Ser(benzyl) and BOC-Gly, again with double coupling. The peptide was deprotected and removed from the resin by stirring the resin in liquid hydrogen fluoride/anisole at −5°C for 90 min. After evaporation, the residue was dissolved in 0.05 M acetic acid, filtered, and lyophilized. Purification was achieved by HPLC on a C-18 μBondapak column eluting with 2% acetonitrile in water containing 0.05% trifluoroacetic acid to afford product (40% yield, specific activity = 1.1 Ci/mmol). Chemical and radiochemical purity was >98%.

Synthesis of fatty acid analogs

All reactions were conducted according to standard practice, under a nitrogen atmosphere. NaH was 50% in a hydrocarbon oil. Temperature was ambient unless otherwise noted. Unless stated otherwise, spectra are reported as follows: NMR, 400 MHz, $CDCl_3$; s = singlet, d = doublet, etc. Infrared spectra (IR) were determined on pure samples (no solvent) unless indicated otherwise. Combustion analyses were conducted by Atlantic Microlab, Atlanta, GA.

9-Oxatetradecanoic acid

Sodium metal (0.30 g, 0.013 mol) was dissolved in dry n-pentanol (100 ml) and then 8-bromooctanoic acid (0.97 g, 4.3 mmol) was added. After stirring overnight and heating at reflux for 24 h, the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and washed with water. The aqueous phase was acidified (pH 1.0, 6 N HCl), extracted with ethyl acetate (3 × 30 ml), dried ($MgSO_4$), and the solvent was removed in vacuo. The resulting yellow oil was chromatographed (silica gel, 0.2% formic acid/CH_2Cl_2) and then subjected to bulb-to-bulb, short path (Kugelrohr) distillation to yield 9-oxatetradecanoic acid (0.4 g, yield = 40%) as a colorless oil (bp 118°C, 0.02 torr). IR: 1720 cm^{-1}; ^{1}H NMR: 0.98 (t, 3H); 1.3 (m, 6H); 1.6 (m, 10H); 2.4 (t, 3H); 3.4 (t, 4H); 10.1 (s, 1H). *Anal.*: calculated for $C_{13}H_{26}O_3$: C, 67.72%; H, 11.32%. Found: C, 67.72%; H, 11.38%.

7,10-Dioxatetradecanoic acid

NaH (2.2 g, 0.045 mol) was allowed to react with 2-butoxyethanol (50 ml) during 1 h. 6-Bromohexanoic acid (4.2 g, 0.0215 mol) was added, the mixture was heated at reflux for 24 h, cooled, and the solvent was removed in vacuo. The residue was dissolved in diethyl ether (50 ml), washed with water (3 × 40 ml), the aqueous phase was acidified (pH 1.0, 6 M HCl), washed with diethyl ether, and the solvent was removed in vacuo. The yellow oil was chromatographed (silica gel, 10–30% diethyl ether/CH_2Cl_2) and distilled (Kugelrohr) to yield the product (2.0 g, 50%, bp 120°C, 0.02 torr). IR: 1740 cm^{-1}. ^{1}H NMR: 0.98 (t, 3H), 1.4 (m, 4H) 1.6 (m, 6H), 2.4 (t, 2H), 3.4 (t, 4H), 3.6 (s, 4H). *Anal.:* calculated for $C_{12}H_{24}O_4$: C, 62.04%; H, 10.41%. Found C, 61.99%; H, 10.47%.

9,12-Dioxatetradecanoic acid

NaH (2.4 g, 0.05 mol) was allowed to react with 2-ethoxyethanol (50 ml) during 30 min. 8-Bromooctanoic acid (4.8 g, 0.021 mol) was added, the solution was heated at reflux for 24 h, cooled, and the solvent was removed in vacuo. The residue was dissolved in ether and washed with water, the aqueous phase was acidified (pH 1, HCl) and washed again with ether. The organic phase was dried ($MgSO_4$), the solvent was removed in vacuo, and the residue was chromatographed (silica gel, 10–25% diethyl ether/CH_2Cl_2), and distilled (Kugelrohr) to afford the title compound (0.4 g, 8%) as a yellow oil (bp 232°C/0.02 torr). IR: 1750 cm^{-1}. NMR: 1.3 (t, 3H); 1.35 (bs, 6H); 1.6 (m, 4H); 3.5 (m, 8H); 10.16 (s, 1H). *Anal.*: calculated for $C_{12}H_{24}O_4$: C, 62.04%; H, 10.41%. Found: C, 61.95%; H, 10.46%.

Ethyl 6,12-dithiatetradecanoate

NaH (0.4 g, 8.4 mmol) was washed with hexanes and then suspended in dry tetrahydrofuran (THF, 55 ml). 5-Ethylthiopentanethiol (8 mmol, 73% pure by gas chromatographic analysis, contaminated by 1,5-bis(ethylthio)pentane, 1.80 g of mixture) in THF (8 ml) was added and stirred for 1 h at room temperature. Ethyl 5-iodovalerate (2.1 g, 8 mmol) in THF (8 ml) was added and the mixture was refluxed for 8 h. The solvent was evaporated in vacuo, the residue was dissolved in ethyl acetate (150 ml), washed with water (2 × 50 ml), brine (50 ml), dried (Na_2SO_4), chromatographed (silica gel, ethyl acetate/hexanes 1:10), and distilled (Kugelrohr) to yield the title compound (1.86 g, 80%) as a colorless oil (bp 147–149°C/0.2 torr). IR: 1740 cm^{-1}. ^{1}H-NMR: 1.25 (t, 6H); 1.50 (quintet, 2H); 1.54–1.67 (m, 6H); 1.73, quintet, 2H); 2.32 (t, 2H); 2.46–2.57 (m, 8H); and 4.12 (q, 2H). *Anal.*: calculated for $C_{14}H_{28}S_2O_2$: C, 57.49%; H, 9.65%. Found: C, 57.36%; H, 9.59%.

6,12-Dithiatetradecanoic acid

Sodium hydroxide (1 M, 6.2 ml, 6.2 mmol) was added to a solution of ethyl 6,12-dithiatetradecanoate (0.9 g, 3.1 mmol) in methanol (15 ml). After stirring for 4 h, water

(20 ml) was added and the reaction mixture was acidified (pH 1, HCl), extracted with ethyl acetate (2 × 100 ml), and the organic phase was washed with water (20 ml), brine (saturated aqueous NaCl solution, 20 ml), and then dried (Na_2SO_4). The product crystallized from n-hexane to yield a white solid (0.77 g, 95%, mp 49.5–50.0°C). IR (KBr): 3000, 1720 cm^{-1}; ^1H NMR, 1.23 (t, 3H), 1.49 (quintet, 2H), 1.56–1.68 (m, 6H), 1.74 (quintet, 2H), 2.38 (t, 2H), 2.51 (t, 2H), 2.53 (t, 4H), 2.54 (q, 2H), and 10.45 (b, 1H). *Anal.*: calculated for $C_{12}H_{24}S_2O_2$: C, 54.50%; H, 9.15%. Found: C, 54.59%; H, 9.21%.

9-Thiatetradecanoic acid

1-Pentanethiol (0.85 g, 8.1 mmol) in THF (8 ml) was added to a suspension of NaH (0.34 g, 8.5 mmol) in dry THF (55 ml) and the mixture was stirred for 1 h at room temperature. Ethyl 8-iodooctanoate (2.42 g, 8.1 mmol) in THF (7 ml) was then added and the reaction mixture was refluxed for 7 h. The same work-up as used for ethyl 6, 12-thiatetradecanoate (see above) gave ethyl 9-thiatetradecanoate (1.96 g, 88%) which appeared as a colorless oil (bp 110–114°C/0.08 torr).

Sodium hydroxide (1 M, 39 ml, 39 mmol) was added to a solution of ethyl 9-thiatetradecanoate (1.5 g, 5.6 mmol) in methanol (20 ml). After stirring for 7 h, the reaction mixture was treated as described for 6,12-dithiatetradecanoic acid to yield the title compound (1.36 g, 99%, mp 35–35.5°C). IR (KBr): 3000, 1710 cm^{-1}; ^1H NMR 0.91 (t, 3H), 1.27–1.43 (m, 10H), 1.53–1.68 (m, 6H), 2.36 (t, 2H), 2.51 (t, 4H), and 10.13 (b, 1H). *Anal.*: calculated for $C_{13}H_{26}SO_2$: C, 63.37%, H, 10.64%. Found: C, 63.29%; H, 10.66%.

8Z-11-Phenylundecanoic acid

7-Carboxyheptyltriphenylphosphonium bromide (ref. 35), mp 111–113°C (lit. mp 104–105°C, in ref. 36, 116–120°C in ref. 35) (9.71 g, 20 mmol) was stirred with potassium t-butoxide (4.91 g, 44 mmol) in THF (70 ml) and hexamethylphosphoric triamide (10 ml) for 30 min. The solution was cooled to 0°C, dihydrocinnamaldehyde (2.68 g, 20 mmol) in THF (20 ml) was added dropwise, and the mixture was stirred for 12 h. The residue was poured into water (150 ml), washed with diethyl ether (50 ml), acidified (pH 1, 2N HCl), and extracted (hexanes, 4 × 50 ml), dried over $MgSO_4$, and the solvent was removed in vacuo. Chromatography (silica gel, 1:1 hexanes–ethyl acetate) and distillation (Kugelrohr) yielded product (2.09 g, 40%) as a colorless oil (bp 136–138°C/0.005 torr). IR 3450–2550 (COOH), 1720 (COOH), 695 (Z-CH=CH) cm^{-1}; ^1H NMR 1.26 (m, 6H); 2.34 (m, 4H); 2.65 (t, 2H); 5.35 (m, 2H); 7.20 (m, 5H); 10.77 (bs, 1H). *Anal.*: calculated for $C_{17}H_{24}O_2$: C, 78.42%; H, 9.29%. Found: C, 78.27%; H, 9.32%. The Z:E ratio (>99:1) was determined by gas chromatography (Varian Aerograph, model 1400, 5 ft × 0.125 in, 10% SE 30 on 60/80 NAW Chromosorb P, 280°C) of the methyl ester (diazomethane).

11-Phenylundecanoic acid

8Z-11-Phenylundecanoic acid (1.30 g, 5 mmol), 5% palladium catalyst (Pd/C, 130 mg) and anhydrous ethanol (50 ml) were shaken under 15 psi H_2 for 1.5 h. The Pd/C was filtered and washed with ethanol. The alcohol was evaporated in vacuo. The residue was crystallized from hexanes to yield white crystals (1.21 g, 91%), mp 42–43°C (mp in ref. 37, 43–44°C), IR: 3400–2500, 1700 cm^{-1}: ^1H NMR 1.27 (m, 12H); 1.57 (m, 4H); 2.30 (t, 2H); 2.55 (t, 2H); 7.20 (m, 5H); 10.58 (b, 1H). *Anal.*: calculated for $C_{17}H_{26}O_2$: C, 77.82%; H 9.99%. Found: C, 77.72%; H, 10.04%.

In vitro NMT assay

Our in vitro NMT assay system has been described in previous papers (19, 20) and is summarized in **Fig. 1**. Acyl CoA thioesters of each analog were generated using the nonspecific *Pseudomonas* CoA ligase (38). To make sure that there was minimal variability in the efficiency of enzymatic conversion of analogs to their CoA esters by this ligase, each analog was screened with the tritiated octapeptide in the presence of a 10-fold range of ligase (0.3–3 units/ml of reaction mixture) and over a 3-fold range of time (25–75 min). With the exception of 7,10-dioxamyristic acid, 0.3 units/ml of ligase and a 25-min incubation were sufficient for maximal production of acylpeptide. For 7,10-dioxamyristic acid, maximal production of acylpeptide occurred either after a 75-min incubation with 0.3 units/ml ligase or a 25-min incubation with 3 units/ml of the enzyme. The later conditions were used for subsequent kinetic studies of this compound.

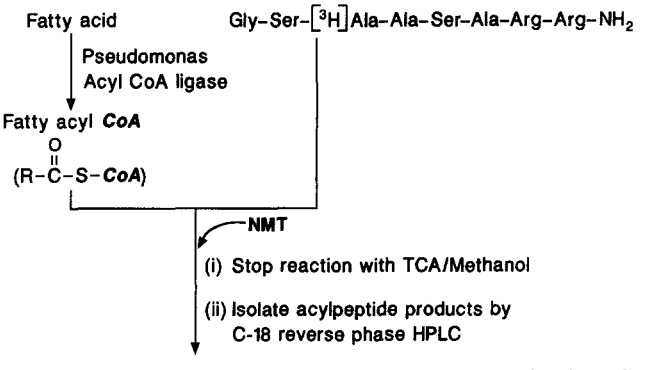

Fig. 1. Summary of the coupled in vitro assay used to examine the acyl CoA and peptide substrate specificities of *S. cerevisiae* NMT. Details of the assay are provided in refs. 19 and 20 and in Experimental Procedures.

As in previous studies (20), we first determined the apparent peptide K_m and V_m using saturating concentrations of analog (15 μM for all the compounds except 9,12- and 7,10-dioxamyristic acid where concentrations of 170 μM (five times its K_m) and 375 μM (three times its K_m) were used). We then determined the acyl CoA K_m and V_m using the peptide substrate at its K_m. At this concentration, *S. cerevisiae* NMT is likely to be 50% saturated for Gly-Ser[^{3}H]Ala-Ala-Ser-Ala-Arg-Arg-NH_2. Kinetic characteristics for myristoyl CoA and each analog were determined in parallel. All V_m data were normalized to the V_m for myristoyl CoA. All experiments were performed at least three times and the data were averaged.

RESULTS AND DISCUSSION

A series of oxygen- and sulfur-substituted, as well as branched chain and aromatic, analogs of myristic acid were produced to address two questions about NMT-acyl CoA interactions. The first had to do with the hydrophobic/hydrophilic requirements of NMT's acyl CoA binding site. Specifically, we wanted to know whether more than one heteroatom substitution could be introduced into the hydrocarbon chain without eliminating its ability to act as substrate for the acyltransferase. If further reductions in hydrophobicity could be achieved in this manner without severely compromising substrate kinetics, then such compounds may more profoundly affect acylprotein structure and/or function (i.e., targeting) than single heteroatom-substituted analogs. The second question was related to the steric requirements of the fatty acyl CoA binding pocket in NMT: how much could we change the bulk of the acyl CoA without profoundly disturbing its substrate kinetics? To address this question, we evaluated branched chain and aromatic fatty acyl CoA esters as NMT substrates.

Previous studies using our C18 reverse phase HPLC system to characterize acylpeptides demonstrated that acylpeptide elution conditions were highly reproducible and depended on both the peptide and acyl chain components. When a single peptide was used, we found that the concentration of acetonitrile required for acylpeptide elution decreased by about 6% for every 2 methylene group decrease in chain length (ref. 20 and inset to **Fig. 2**). The change in acetonitrile concentration required for C18 reverse phase HPLC elution correlated with changes in hydrophobicity as measured by octanol/water partition (correlation coefficient, 0.907). As demonstrated in Fig. 2 (and ref. 20), single sulfur or oxygen for methylene substitutions lead to reductions in the acetonitrile concentration required for elution comparable to those expected for dodecanoyl (50% acetonitrile) and decanoyl (45%) peptides, respectively. Double sulfur or oxygen for methylene substitutions lead to twice the change in hydrophobicity so that the concentration of acetonitrile required for acylpeptide elution of 6,12-dithiamyristoyl peptide (43–44%) or either of the dioxamyristoyl peptides (32–35%) is roughly that expected for decanoyl- or hexanoyl-peptides, respectively (see insert in Fig. 2). Thus, substitution of oxygen or sulfur for methylene groups in the fatty acyl chain leads to dramatic reductions in hydrophobicity with minimal alteration in chain length or stereochemistry.

Analogs were tested as potential substrates for NMT using a coupled in vitro assay system. In this system, free fatty acids are first enzymatically converted to CoA esters using a large excess of the relatively nonspecific *Pseudomonas* acyl CoA ligase (38). A radiolabeled peptide substrate (Gly-Ser-[^{3}H]Ala-Ala-Ser-Ala-Arg-Arg-NH_2) and partially purified (150-fold) *S. cerevisiae* NMT are then added to the reaction mixture. The acylpeptides produced are subsequently purified by C18 reverse phase HPLC and quantitated by scintillation counting.

Given the fact that this is a coupled enzyme system, we attempted to control for variability in the extent of conversion of fatty acid analogs to their acyl CoA esters as described in Experimental Procedures. In earlier work (20) we compared the efficiency of conversion of two naturally occurring radiolabeled fatty acids ([^{3}H]myristic and palmitic acids) and a heteroatom-containing analog ([^{3}H]11-oxamyristic acid) by *Pseudomonas* CoA ligase. These studies revealed that ~50% of each compound was converted to its respective CoA ester. Since the compounds synthesized for the studies reported here were not radiolabeled (and conversion to CoA ester could not be directly assessed), we felt that it was important to demonstrate empirically that the rate of acylpeptide formation is

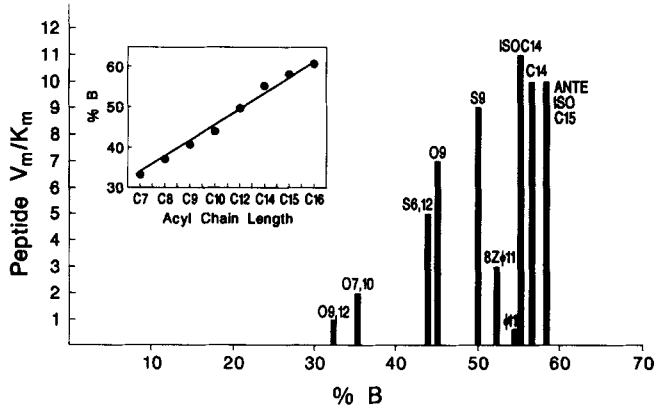

Fig. 2. Peptide V_m/K_m versus % B. Peptide catalytic efficiency (V_m/K_m) with different acyl CoA analogs is plotted against the percentage of acetonitrile required for elution of the acylpeptide from our C18 reverse phase HPLC system. (B, acetonitrile). The inset shows the relationship between elution time and the acyl chain length of standards each containing the octapeptide Gly-Ser-[^{3}H]Ala-Ala-Ser-Ala-Arg-Arg-NH_2 (linked to C7:0–C16:0).

TABLE 1. Characterization of fatty acid analogs

Analog	Peptide K_m μM	Peptide V_m	AcylCoA K_m μM	AcylCoA V_m	Name
$CH_3(CH_2)_{12}COOH$	10	100%	0.6	100%	myristic acid
$CH_3(CH_2)_4O(CH_2)_7COOH$	23	155%	1.8	260%	9-oxamyristic acid
$CH_3(CH_2)_4S(CH_2)_7COOH$	12	110%	0.12	100%	9-thiamyristic acid
$CH_3CH_2OCH_2CH_2O(CH_2)_7COOH$	123	134%	34	195%	9,12-dioxamyristic acid
$CH_3(CH_2)_3OCH_2CH_2O(CH_2)_5COOH$	75	147%	125	58%	7,10-dioxamyristic acid
$CH_3CH_2S(CH_2)_5S(CH_2)_4COOH$	44	200%	2.4	126%	6,12-dithiamyristic acid
$CH_3CH(CH_2)_{10}COOH$ (CH3 branch)	26	280%	2.7	530%	isomyristic acid
$CH_3CH_2CH(CH_2)_{10}COOH$ (CH3 branch)	19	190%	3	310%	anteisopentadecanoic acid
C_6H_5-$(CH_2)_{10}COOH$	67	39%	1.6	4%	11-Phenylundecanoic acid
C_6H_5-$(CH_2)_2CH=CH(CH_2)_6COOH$	31	100%	2.9	15%	8Z-11-Phenylundecanoic acid

Details of the methods used to determine these peptide and acyl CoA K_m and V_m data are described in Experimental Procedures.

not limited by either the amount of CoA ligase or the length of pre-incubation used to generate acyl CoA esters. Concern about the efficiency of conversion largely applies to analogs with high K_m values, since the high K_m could reflect poor binding to the acyltransferase or inefficient conversion to their CoA derivatives. As detailed in the Experimental Procedures section, the time and acyl CoA ligase concentration used were determined empirically for each analog to allow maximal acylpeptide synthesis. It is important to emphasize that the coupled in vitro assay offers a means for comparing the kinetic properties of analogs with respect to one another and with those of myristic acid. Studies using *S. cerevisiae* NMT and this in vitro assay system have accurately predicted the patterns of in vivo incorporation of myristic acid, 11-oxamyristic acid, and 13-oxamyristic acid into mammalian (mouse, rat, and human) cellular N-myristoylproteins (ref. 24 and data not shown).

Multiple heteroatom-substituted analogs of myristic acid can serve as substrates for NMT

The peptide kinetic data presented in **Table 1** indicate that when myristoyl CoA is used as an NMT substrate, Gly-Ser-[^{3}H]Ala-Ala-Ser-Ala-Arg-Arg-NH_2 has a relatively high apparent affinity for the acyltransferase (K_m = 10 μM, see footnote 3) and myristoylpeptide is efficiently generated (V_m normalized to 100%). Myristoyl CoA is even more tightly bound by NMT (K_m = 0.6 μM) than the peptide substrate.

Myristic acid analogs with either a sulfur or oxygen substitution at position 9 in the fatty acid backbone are good NMT substrates. When 9-thiatetradecanoic acid (9-thiamyristic acid) is added to the coupled in vitro assay system, the apparent affinity of NMT for the [^{3}H]octapeptide substrate (K_m = 12 μM) and the rate of acylpeptide formation (V_m = 110%) is comparable to that seen with myristic acid. Peptide catalytic (V_m/K_m) efficiency with 9-thiamyristoyl CoA is 90% of that with myristoyl CoA (see Fig. 2). 9-Thiamyristoyl CoA also appears to be bound to NMT with slightly greater affinity than myristoyl CoA (K_m = 0.12 μM vs 0.6 μM), giving an acyl CoA catalytic efficiency even better than that of myristoyl CoA. When 9-oxatetradecanoic acid (9-oxamyristic acid) is introduced into the reaction mixture, the calculated K_m of the [^{3}H]octapeptide increases only about 2-fold compared to myristic acid, but the rate of acylpeptide formation is also slightly higher, producing a peptide catalytic efficiency 70% of that with C14:0 (see Fig. 2). This single oxygen-substituted analog is bound to NMT with a slightly lower apparent affinity (K_m = 1.8 μM, Table I) than is myristoyl CoA, but acyl CoA catalytic efficiency remains 86% of myristoyl CoA (**Fig. 3**). Thus, in agreement with previous studies of analogs with single sulfur or oxygen for methylene substitutions at other positions in the backbone (20), these two compounds are efficiently utilized as substrates by *S. cerevisiae* NMT in vitro.

We next determined whether further reductions in hydrophobicity could be achieved without severely compromising acylCoA or peptide catalylic efficiency. We therefore synthesized three compounds with multiple heteroatom substitutions: 6,12-dithiamyristic acid, 9,12 dioxamyristic acid, and 7,10-dioxamyristic acid. The double sulfur-substituted analog 6,12-dithiamyristoyl CoA is efficiently transferred to the synthetic octapeptide substrate (200% of myristoyl CoA, Table 1) but has a significantly higher K_m than myristoyl CoA (2.4 μM) and causes a re-

[3]Note that these are *apparent* K_m values calculated from double reciprocal plots of data generated using the coupled in vitro assay system (19). The peptide Gly-Asn-[^{3}H]-Ala-Ala-Ser-Ala-Arg-Arg-NH_2 was selected for these studies because its relatively high V_m and low K_m allow the use of small amounts of radiolabeled peptide for each assay. More than 95 peptides have been evaluated in this assay system with myristoyl CoA (ref. 1) and 7 peptides have been evaluated with radiolabeled 10-(propoxy)decanoyl CoA (24).

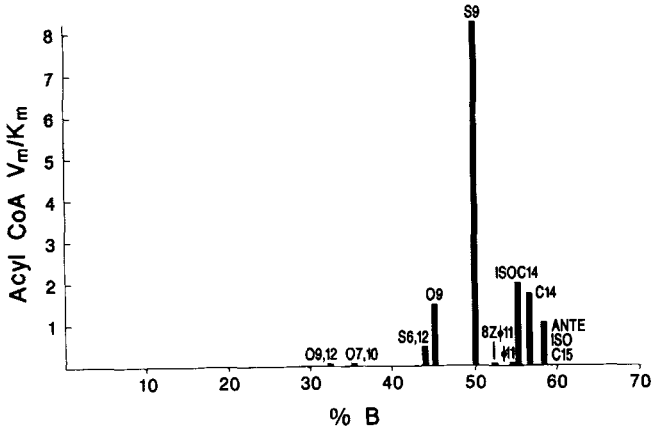

Fig. 3. Acyl CoA V_m/K_m versus % B. The catalytic efficiency (V_m/K_m) for various acyl CoA analogs is plotted against the percentage of acetonitrile required for acylpeptide elution from our C18 reverse phase HPLC system (B, acetonitrile).

duction in apparent affinity of NMT for the peptide substrate (44 μM). The peptide kinetic characteristics observed with 6,12-dithiamyristic acid are, however, quite "reasonable", given the peptide kinetic data for the corresponding single sulfur-substituted analogs (20): *i*) the peptide K_m with 12-thiamyristoyl CoA is about 2-fold higher than that with myristoyl CoA, and *ii*) the peptide V_m for 6- and 12-thiamyristoyl CoA are 213% and 98% of those observed with myristoyl CoA, respectively (20).

By contrast, the double oxygen-substituted analog, 9,12-dioxamyristic acid, is a much poorer NMT substrate than might be expected from kinetic analysis of analogs having single oxygen substitutions in the same positions. The peptide K_ms with 9-oxamyristoyl CoA and 12-oxamyristoyl CoA are ~2-fold and 1.5-fold higher than with myristoyl CoA, respectively. The peptide K_m with 9,12-dioxamyristoyl CoA (123 μM, Table 1) is, however, ~12-fold higher than that with myristoyl CoA (10 μM). 7,10-Dioxamyristoyl CoA is also a poor NMT substrate giving a peptide K_m ~8-fold higher (75 μM, Table 1) than that obtained with myristoyl CoA. In addition, both of these double oxygen-substituted analogs are poorly bound by NMT. The relatively inefficient use of these substrates by NMT is shown graphically in Figs. 2 and 3. In support of our previous data (20), these analogs demonstrate that the acyl CoA bound to NMT may profoundly influence NMT-peptide interactions. They also suggest that properties other than chain length and stereochemistry are important for determining which acyl CoA esters will serve as NMT substrates.

At this point, the precise nature of the molecular recognition that produces the substrate properties of double oxygen- and double sulfur-substituted analogs remains unclear. Sulfur- and oxygen-containing myristic acid analogs differ relatively little in size from myristic acid as judged by CPK molecular models. The CPK models visually reflect van der Waals volumes. Another measure of size are the so-called A-values (39, 40). These are a measure of steric interference reflected in 1,3-diaxial interactions in chair-form cyclohexane derivatives. Sulfur is expected to have a larger volume than oxygen but its longer bond distances compared to oxygen make the overall steric effects similar, at least as measured using these values. Sulfur and oxygen do, however, differ significantly in electronegativity (oxygen = 3.5; sulfur = 2.5) and, correspondingly, in their ability to accept a hydrogen bond. Water or other hydrogen bonding solvent is expected to be more organized by an oxygen atom than by sulfur. It is uncertain at present what role these properties play in what is believed to be an interaction between a hydrophobic binding pocket and a hydrophobic fatty acyl chain. These questions will be further explored using a strategy based on comprehensive analog synthesis. Detailed structural analysis of NMT should also help us to understand the acyl CoA and peptide interactions with this enzyme.

Exploration of the steric requirements of NMT for its acyl CoA substrates

To examine the influence of steric bulk on acyl CoA interactions with NMT, we initially tested two related branch chain fatty acids, isomyristic acid and anteisopentadecanoic acid. Isomyristic acid is a 13-carbon chain length fatty acid with a branching methyl group at position 12. Anteisopentadecanoic acid is a myristic acid (14-carbon chain length) derivative with a methyl group replacing the proton at position 12. Fig. 2 shows that the acetonitrile concentration required for elution of these acylpeptides is comparable to that of the myristoylpeptide (55–58%). When their acyl CoA esters are bound to NMT, the synthetic peptide substrate is still bound with high affinity and acyl peptides are efficiently generated; the peptide V_m/K_m associated with these two compounds is virtually identical to that of myristic acid (see Table 1 and Fig. 2). These results suggest that the conformation of NMT required for catalysis is only minimally perturbed by its interaction with these branch chain acyl CoA esters. One possible interpretation of these results is that the fatty acyl chain binding site resembles a "groove" or "crevice" on NMT rather than a "hole." The "extra" methyl group may then reside along the path that the adjacent methylene group normally follows to enter the acyl chain binding site. Interestingly, although the acyl CoA apparent K_m is significantly higher than that of myristoyl CoA for both these analogs, V_m is also dramatically elevated (Table 1 and Fig. 3) suggesting that the orientation of the acyl CoA and peptide in NMT's catalytic site is actually better for acylpeptide synthesis with these branched chain analogs than with myristoyl CoA.

To further define the limits of steric bulk for NMT substrates, we synthesized two compounds containing aromatic rings: 8Z-11-phenylundecanoic acid and 11-phenylundecanoic acid. These fatty acid analogs are comparable in chain length and hydrophobicity to myristate but have a rigid, planar ring in place of the normally flexible alkyl chain. These compounds differ from each other only by the presence of a *cis* double bond between carbons 8 and 9. Both aromatic analogs are relatively poor NMT substrates (Table 1 and Figs. 2 and 3). The striking finding, however, is that the Δ8 *cis* unsaturated acyl chain is a significantly better NMT substrate than the saturated analog. Peptide K_m is lower with the Δ8 *cis* unsaturated acyl chain, and the rate of acyl peptide formation is higher, producing a 5-fold difference in peptide catalytic efficiencies between the two aromatic compounds (Fig. 2).

The ability of NMT to accommodate aromatic fatty acid analogs (albeit poorly) raises the possibility of creating photoaffinity fatty acid analogs. These compounds could, in turn, be used for both structure/function studies of NMT and to map the interaction of acyl chains on specific N-myristoylproteins with other cellular macromolecules. The observation that the *cis* Δ8 unsaturated aromatic analog is a significantly better substrate than the corresponding saturated aromatic fatty acid supports our previous observation (20) that restricting the stereochemistry of the fatty acyl chain can significantly improve or reduce the efficiency of NMT-substrate interactions. The combination of heteroatom substitution and unsaturation may therefore allow the development of high affinity acyl CoA substrates which differ dramatically from myristoyl CoA in their physical-chemical properties.

As noted above, previous studies have demonstrated the ability of the in vitro NMT assay to predict the behavior of the enzyme in vivo. These studies also indicated that it is the combination of acyl CoA and peptide substrate which is crucial for determining the efficiency of acylpeptide formation. The in vitro data presented here suggest that a wide variety of analogs with differing physical-chemical properties may serve as substrates for NMT. Thus, one possibility arising from these results is that we may be able to systemically alter the nature of the acyl chain of specific subsets of N-myristoylproteins in vivo by exposing cells to these alternative substrates. This may represent a powerful method for examining the role of acyl chain structure in protein function. If the myristoyl moiety is involved in protein-protein interactions or protein folding, it is expected that different structural classes of fatty acid analogs will influence the function of specific proteins in different ways. Our preliminary observations (28) that different heteroatom-containing analogs of myristic acid affect the assembly of HIV-I and MoMLV appear to support this hypothesis.

We wish to thank Luis Glaser for his many valuable suggestions and Sean Nugent for his help in synthesizing 9-thiamyristic acid. This work was supported by grants from the National Institutes of Health (AI-27179) and Monsanto Company. R.O.H. was supported by a Spencer Olin Foundation and Gerty T. Cori Fellowships and the Medical Scientist Training Program (GM-07200). J. I. G. is an Established Investigator of the American Heart Association.

Manuscript received 14 December 1989 and in revised form 20 February 1990.

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