We have explored the acyl-CoA substrate specificity of Saccharomyces cerevisiae myristoyl-CoA:protein N-myristoyltransferase (NMT) by synthesizing 81 fatty acid analogs and surveying their activity in a coupled in vitro assay containing Pseudomonas acyl-CoA synthetase and Escherichia coli-derived yeast NMT. Single oxygen or sulfur substitution for C-3 with E6. Control experiments indicated that this apparent isomerization was not due to the Pseudomonas acyl-CoA synthetase present in the coupled assay system. Together the data suggest that the acyl chain of myristoyl-CoA is present in a bent conformation in the acyl-CoA-binding site of S. cerevisiae NMT. The site appears to possess a complex measuring device that can sense distance along the chain from carboxyl to the ω end of the fatty acid as well as steric volume at the ω terminus. The overall shape of this sensor may be conical with the cone and the terminal carbons coaxial. In this proposed model, the relative sensitivity to chain length versus steric volume would be determined by the acuteness of the “cone.”

Myristoyl-CoA:protein N-myristoyltransferase (NMT; 3.5.1.97) catalyzes the co-translational (Wilcox et al., 1988) attachment of myristate (C14:0) to the N-terminus of a protein. The site of attachment is the acyl-CoA-binding site of the enzyme. The enzyme is a dimeric protein with two active sites per subunit. Each active site is composed of a catalytic domain and an acyl-CoA-binding domain. The catalytic domain contains the myristoyl-CoA binding site and the myristoyl-CoA transferase activity. The acyl-CoA-binding domain is responsible for binding the acyl-CoA substrate and for transferring the myristoyl-CoA to the protein substrate. The interaction between the acyl-CoA substrate and the enzyme is mediated by hydrogen bonding and hydrophobic interactions. The acyl-CoA substrate is a hydrophobic molecule that is stabilized by the hydrophobic environment of the enzyme. The acyl-CoA substrate is recognized by a hydrophobic pocket in the enzyme that is complemented by the hydrophobic acyl-CoA substrate. The acyl-CoA substrate is then transferred to the protein substrate by a nucleophilic attack on the thioester bond of the acyl-CoA substrate. The acyl-CoA substrate is released from the enzyme as free myristoyl-CoA.
NH₂-terminal glycine residue of a variety of cellular, viral, and oncoproteins (reviewed in Towler et al., 1988). The functional significance of this covalent protein modification has been assessed in two ways: (i) prohibition of N-myristoylation by deletion or substitution of the acceptor Gly residue using site directed mutagenesis, and (ii) the use of myristic acid analogs with altered physical-chemical properties that are substrates for cellular acyl-CoA synthetase and NMT (Heuckeroth et al., 1988a, 1988b, 1990; Heuckeroth and Gordon, 1989; Johnson et al., 1990). For example, substitution of the Gly residue of the Pr56gr polyprotein precursor of human immunodeficiency virus 1 (HIV-1) with an Ala, prevents its N-myristoylation, blocks its proteolytic processing, and profoundly inhibits viral replication (Gottlinger et al., 1989; Bryant and Ratner, 1990). When a CD4 human lymphoid cell line (H9) is acutely or chronically infected with HIV-1 and treated with oxygen- or sulfur-substituted analogs of myristate that are substrates for NMT, viral assembly is inhibited without accompanying cellular toxicity (Bryant et al., 1989, 1991).

These single heteroatom for methylene group substitutions result in significant reductions in hydrophobicity, equivalent to the loss of 2-4 methylene groups (Heuckeroth et al., 1988b). Metabolic labeling studies have shown that oxygen substituted analogs enter Saccharomyces cerevisiae as well as mammalian cells and are selectively transferred by NMT into subsets of cellular N-myristoyl proteins (Heuckeroth and Gordon, 1989; Johnson et al., 1990; Mumbry et al., 1990). This observation is consistent with the results of previous in vitro studies using naturally occurring fatty acids of varying chain lengths as well as a limited number of heteroatom substituted analogs (Heuckeroth et al., 1988b). These in vitro analyses employed S. cerevisiae NMT, octapeptide substrates derived from the NH₂-terminal sequences of known N-myristoyl proteins, and an acyl-CoA generating system. They revealed that the enzyme is able to monitor acyl-CoA length and that cooperative interactions occur between the enzyme's acyl-CoA and peptide-binding sites.

The gene encoding S. cerevisiae NMT has been cloned (Duronio et al., 1989). The 455-residue monomeric (Towler et al., 1987) protein has been efficiently expressed in Escherichia coli and subsequently purified to apparent homogeneity (Duronio et al., 1989; Rudnick et al., 1990). The interactions between NMT and its acyl-CoA and peptide ligands have been monitored using a variety of biophysical methods. They indicate that an acylenzyme intermediate can form involving a domain spanning Arg⁴⁵ to Thr⁷⁰ and suggest that the mechanism of catalysis may be ordered, preferred ordered, or ping pong (Rudnick et al., 1990).

One of our major goals is to characterize the enzyme's acyl-CoA-binding pocket in the hope that such information will permit design of active analogs, having altered physical-chemical properties, which can be selectively targeted to specific N-myristoyl proteins. Such analogs cannot only be used to explore issues of molecular recognition by the enzyme but also the functional importance of the myristoyl moiety in a given N-myristoyl protein (e.g. Johnson et al., 1990; Bryant et al., 1989, 1991; Mumbry et al., 1990). We have now prepared 81 analogs that have allowed us to systematically explore the effects of varying several physical chemical properties of the fatty acid on its recognition by the acyl-CoA-binding site of S. cerevisiae NMT.

**EXPERIMENTAL PROCEDURES²**

**Synthesis of Oxygen-containing Analogs of Myristic Acid—**The syntheses of the single oxygen-containing analogs were accomplished by four separate approaches. The simplest preparation was to treat an ω-halocarboxylic acid with the appropriate alkoxy base:

\[
H(CH₃)₂OH + X-(CH₂)m-COOH → H(CH₃)₂O-(CH₂)mCOOH
\]

This approach was used whenever possible and was successfully employed for the following analogs: 3-oxatetradecanoic acid (O-3), 5-oxatetradecanoic acid (O-5), 9-oxatetradecanoic acid (O-9), 11-oxatetradecanoic acid (O-11), and 13-oxatetradecanoic acid (O-13).

8-Oxatetradecanoic acid (O-8) and 12-oxatetradecanoic acid (O-12) were synthesized as the corresponding nitrides and then hydrolysed. 4-Oxatetradecanoic acid (O-4) was produced by cyanomethylation of n-decanol followed by hydrolysis:

\[
H(CH₃)₂OH + CH₂=CH-CN →
\]

\[
H(CH₂)₄=CH₂CH₂CN → H(CH₂)₄O-(CH₂)mCOOH
\]

7-Oxatetradecanoic acid (O-7) and 10-oxatetradecanoic acid (O-10) were synthesized by converting an alcohol into an alkyl bromoalkyl ether followed by condensation with diethyl malonate and subsequent decarboxylation hydrolysis:

\[
C₆H₁₂O + Br-CH₂=CH-Br → C₆H₁₂OC₂H₄Br →
\]

\[
C₆H₁₂O-(CH₂)mCOOH
\]

6-Oxatetradecanoic acid was difficult to obtain and is the subject of a separate report (Katoh et al., 1991).

**Synthesis of Sulfur-containing Myristic Acid Analogs—**Most of the single-sulfur-substituted derivatives (S-5, S-6, S-7, S-8, S-9, and S-12) were prepared by treating the appropriate alkane thiol with an ω-halocarboxylic acid ester or nitride followed by hydrolysis. Cyanomethylation of decanethiol yielded S-4 after hydrolysis. The general procedure is illustrated below:

\[
H(CH₂)₄-SH + X-(CH₂)m-Y →
\]

\[
H(CH₂)₄-S-(CH₂)m-Y → H(CH₂)₄S-(CH₂)mCOOH
\]

The converse of this procedure was used to obtain 13-thiatetradecanoic acid (S-13): 12-mercaptododecanoic acid was methylated (CH₃I) to give S-13. S-3 was prepared by a method previously reported in the literature (Smith and Hernestam, 1951).

Full details of the syntheses, purification, and characterization of these single heteroatom analogs as well as analogs having multiple oxygen and/or sulfur substitution may be found in the Miniprint.

**Synthesis of Aromatic Analogs—**Information concerning the syntheses and chemical characterization of analogs used in this study is provided in the Miniprint. Compound structures and names are itemized in Table III.

**Synthesis of E, Z, and Y Isomers of Myristate—**Details concerning the syntheses of these 26 analogs may be found in the Miniprint. The triple bond compounds (designated Y for yne) were prepared by alkylation of a deprotonated terminal alkyne where possible. Z (cis) isomers were generally prepared by a Wittig reaction in which the phosphonium salt arose from an ω-halocarboxylic acid. This, in turn, was treated with the appropriate aldehyde. The few variations from these procedures are described further in the Miniprint. The three E (trans) isomers of tetradecanoic acid were prepared from the corresponding alkyne derivatives. Isoener purity was assessed in all cases by gas chromatographic and NMR analyses (see below).

**Synthesis of Radiolabeled Octa-Lysines—**Gly-Ala-Ara-[H]Ala-Ser-Val-Leu-Ser-NH₂(GAR-[H]AVLS-NH₂), representing residues 2-9 of the human immunodeficiency virus 1 Pr55⁵⁵⁵⁵ (Ratner et al., 1985), was synthesized using a four-step procedure. 1) The Ser-Ser² fragment was synthesized on p-methylbenzhydrylamine (mBHA) resin with an Applied Biosystems model 430A peptide synthesizer using Boc-protected L-αmino acids and double coupling synthesis cycles. 2) t-Boc-3-[¹⁴C]l-α-alanine was prepared by reacting [⁳⁻¹⁴C]l-α-alanine (4

² Portions of this paper (including part of "Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Substrate Specificity of N-Myristoyltransferase

FIG. 1. In vitro assay of Pseudomonas acyl-CoA synthetase activity. Panel A shows the reverse-phase HPLC elution profile of coenzyme A (CoA), myristoyl-CoA (MyrCoA), and myristate (Myr) from the C8 column. Authentic radiolabeled standards were used. Panels B and C demonstrate the relationships between the amount of [3H]myristoyl-CoA produced during a 25-min incubation and the amount of myristate and enzyme added to the reaction mixture.

mg, 44.7 μmol, 1.45 Ci/mmol) with di-t-butyl dicarbonate (133 mg, 605 μmol) in 1:1 dioxane/water (0.6 ml) containing 1 N NaOH (0.2 ml) for 15 min. The t-Boc-[3H]H-alanine was then coupled to resin-bound Ser2-Ser peptide (61 mg of resin, 36 μmol of peptide) with amination for 48 h in a silanized glass reaction vessel. Subsequently, the peptide resin was reacted a second time with excess unlabeled t-Boc-alanine to maximize incorporation at the fourth position. The peptide resin was then washed in sequence by double coupling appropriate t-Boc amino acids to the peptide resin. 4) The labeled peptide was cleaved from the resin and deprotected with HF/anisole (9:1) for 60 min at 0 °C and purified by high performance liquid chromatography (HPLC). The purified product had a specific activity of 1.2 Ci/mmol (total yield, 28 mCi). Radiochemical and chemical purity was greater than 95% as ascertained by analytical HPLC.

The synthesis of Gly-Ser-[3H]Hala-Ala-Ser-Ala-Arg-Arg-NH2(GS-[3H]AASARR-NH2 specific activity 1.1 Ci/mmol) is described in a previous publication (Heuckeroth et al., 1988b). Gly-Ser-[3H]AASARR-NH2-[35S]Tyr-Arg-Arg-NH2(GNAS[125I]YRR-NH2, specific activity 8,800 cpm/pmol) was prepared and purified according to Heuckeroth et al. (1988b).

In Vitro Pseudomonas Acyl-CoA Synthetase Assay—Generally labeled [14C]CoA was prepared by exposing 100 mg of the compound to tritium gas for 14 days at room temperature (Du Pont-New England Nuclear). The material was then washed with water and purified by C8 reverse-phase HPLC (specific activity 1.2-1.5 Ci/mmol).

The efficiency of conversion of analogs to their corresponding CoA thioesters by Pseudomonas CoA synthetase was determined as follows. A 100-μl reaction mixture containing fatty acid (final concentration = 160 μM), 1 mM CoA, 1 μCi of [3H]CoA, 5 mM ATP, 3 mM dithiothreitol (DTT), 0.05% Triton X-100, 5 mM Tris, pH 7.4, 2.5 mM MgCl2, 50 μM EGTA, and 0.03 units of acyl-CoA synthetase (Sigma) was incubated for 25 min at 30 °C. The reaction was quenched with an equal volume of ethanol/acetic acid (1:1), cooled on ice for 5-10 min, and fractionated by C8 reverse-phase HPLC (Waters μBondapak, 3 × 30 cm) using a linear gradient of 0.05 M sodium phosphate, pH 5.5 (buffer A), and acetonitrile (buffer B) at a flow rate of 2 ml/min (the gradient was developed from 20% to 70%). In the case of more hydrophilic analogs with multiple oxygen/sulfur substitutions, an initial shallow gradient was run from 0 to 20% B (2 ml/min over 20 min). The tritiated products were quantitated using an in-line detector (Radiomatic, model A250). Parallel reactions were run using myristic acid.

The reaction readily separated CoA from myristoyl-CoA and myristic acid (Fig. 1A). Generation of myristoyl-CoA was dependent upon the inclusion of ATP and the Pseudomonas acyl-CoA synthetase (data not shown) and was proportional to the concentrations of unlabeled myristate and synthetase in the reaction mixture (Fig. 1, B and C). The rate of production of acyl-CoA by the Pseudomonas enzyme varied as a function of saturated fatty acid chain length (Fig. 2A). Analysis of C7 to C16 fatty acid (FA) substrates revealed an optimal chain length of 10-12 carbons. Under the assay conditions used C14:0 produced approximately 50% of the amount of product observed for C16:0 (Fig. 2A). Approximately 50% of myristate added to the reaction was converted to myristoyl-CoA after a 25-min incubation (data not shown). Fig 2B shows that elution of H-(CH2)4COO- from the C8 column was dependent upon chain length and acetonitrile concentration.

Coupled in Vitro NMT Assay—This in vitro NMT assay has been described in previous papers (Towler and Glaser, 1986; Towler et al., 1987; Heuckeroth et al., 1988b). An initial single point screening survey was conducted as follows. Myristic acid or its analogs (final concentration = 15 μM) was converted to its corresponding CoA derivative using 0.3 units/ml of Pseudomonas acyl-CoA synthetase (Shimizu et al., 1980). Following a 25-min incubation at 30 °C, S. cerevisiae NMT (0.5 μg/ml, purified to apparent homogeneity from E. coli, Rudnick et al., 1989) was added together with either 1H peptide (final concentration 25 μM in the 100-μl reaction mixture). When GNAS[125I]YRR-NH2 was used, its final concentration was 40 μM, and the amount of NMT added was increased to 2.0 ng/μl. After an additional 10 min of incubation at 30 °C, the enzymatically generated, radiolabeled acyl-peptide was purified by C8 reverse-phase HPLC (see Heuckeroth et al., 1988b, 1990) and quantitated using the in-line Radiometric detector. The amount of analog peptide produced was compared to the amount of myristoyl peptide produced in a parallel reaction. All results represent at least duplicate assays.

Selected compounds were subjected to more detailed kinetic analysis. As in previous studies (Heuckeroth et al., 1989b, 1990), we first determined the apparent peptide Km, and Vmax using saturating concentrations of analog. We then determined the apparent acyl-CoA Km, and Vmax using the peptide substrate at its Ks. At this concentration, S. cerevisiae NMT is likely to be 90% saturated with octapeptide. Myristic acid was used as a control in each experiment. All Vmax data were normalized to the Vmax obtained with myristoyl-CoA. All experiments were performed at least twice and the data were averaged.

X-ray Studies of 5-Thiatetradecanoic Acid—Crystals of S-S suitable for x-ray analysis were obtained from hexane (melting point 45-46 °C). The analysis was performed on an Enraf-Nonius CAD-4F diffractometer using a graphite monochromator (radiation, CuKα). The crystal was sheet-like with approximate dimensions 0.03 × 0.68 mm, triclinic, space group P1, α = 5.494 (1), b = 8.032 (2), c = 33.672 (6) Å, α = 147.82 (2) °, β = 94.88 (2), γ = 92.18 (2), V = 9.075 (2), Z = 1, 5418 A, Z = 4, D = 1.111 g cm−3, total number of reflections and unique reflections, 3771 and 1034, respectively, R = 0.0986, R = 0.986.

RESULTS AND DISCUSSION

Replacement of Methylene Groups by Group 6B Heteroatoms—We prepared a panel of analogs in which each methylene (−CH2−) group from position 3 to position 13 of tetradecanoic acid was replaced by either sulfur or oxygen. We did not attempt to produce analogs with substitutions at either the 2 or 14 positions since the resulting hydroxyl, thio, carbonate, or thio carbonate derivatives would be expected to have dramatically different physical and reactivity properties compared to all of the other tetradecanoic acid derivatives. A more limited number of analogs having more than one oxygen, more than one sulfur, and combinations thereof were also prepared. This latter group permitted us to assess the interplay of heteroatoms within the same fatty acid.3

3 Examples of analogs described in this paper and the numbering system are as follows: O=O, CH2(OH)2, O=O(CH2)5−COOH, CH3−CH=CH−(CH2)5−COOH; E or Z7, CH2≡CH−CH=CH−(CH2)5−COOH, and Y5, CH2(CH2)5−C≡C−(CH2)5−COOH.
Replacement of methylene by either oxygen or sulfur will alter the fatty acid in several ways. The most obvious difference between methylene and a group 6B (of the periodic table) heteroatom is that hydrogen atoms are present in the former but not in the latter. Thus, the bond interactions that influence hydrocarbon conformation are reduced by insertion of O or S in their stead. On the other hand, incorporation of a heteroatom introduces new bond angles, different bond distances, and significantly altered electronic effects. Some of the anticipated differences are summarized in Table I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>C</th>
<th>O</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond distance (C-X)</td>
<td>1.54</td>
<td>1.41</td>
<td>1.81</td>
</tr>
<tr>
<td>Bond angle (C-X-C)</td>
<td>109°28'</td>
<td>111°43'</td>
<td>99.1°</td>
</tr>
<tr>
<td>Electronegativity (Pauling)</td>
<td>2.5</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Electronegativity (Sanderson)</td>
<td>2.746</td>
<td>3.954</td>
<td>2.957</td>
</tr>
<tr>
<td>A-value for –X-CH₃</td>
<td>1.7</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Average C-X bond strength</td>
<td>83-85</td>
<td>85-91</td>
<td>68</td>
</tr>
<tr>
<td>First ionization potential</td>
<td>260</td>
<td>314</td>
<td>239</td>
</tr>
<tr>
<td>Dipole moment, C–X</td>
<td>0</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Note that in S-5A and S-5B, the three-carbon unit from C-2 to C-4 is nearly gauche-gauche while C-3 and C-4 are essentially eclipsed in S-6B.

The two conformations of S-5 are illustrated in Fig. 3. The structure of 5-thiatetradecanoic acid as defined by x-ray diffraction studies. Two conformations were defined and are designated S-5A and S-5B. Note that in S-5A, C-2–C-4 is nearly gauche-gauche while C-3 and C-4 are essentially eclipsed in S-6B.

Fig. 2. Conversion of naturally occurring saturated and group 6B-substituted fatty acids to their CoA derivatives by Pseudomonas acyl-CoA synthetase. Details of the in vitro assay are provided under “Experimental Procedures.” Panel A illustrates the chain length specificity of the synthetase. C7:0–C16:0 FA were assayed for their conversion to acyl-CoAs. Panel B demonstrates the linear relationship between the chain length of C7:0–C16:0 FA (indicated by the closed boxes) and the concentration of acetonitrile (%B) required to elute their CoA derivatives from the C18 column. Superimposed on this plot is the %B needed to elute various analogs to their CoA derivatives by x-ray diffraction studies. Two conformations were defined and are designated S-5A and S-5B, respectively. In S-5A, the three-carbon unit from C-2 to C-4 is nearly gauche-gauche while C-3 and C-4 are essentially eclipsed in S-6B. See text and “Experimental Procedures” for further details.

Fig. 3. The structure of 5-thiatetradecanoic acid as defined by x-ray diffraction studies. Two conformations were defined and are designated S-5A and S-5B. Note that in S-5A, C-2–C-4 is nearly gauche-gauche while C-3 and C-4 are essentially eclipsed in S-6B. See text and “Experimental Procedures” for further details.

X-ray diffraction studies were used to gain direct insights into the structural alterations that accompany replacement of CH₂ by O or S in myristic acid. Our efforts were limited by the fact that all of the analogs described in this paper have melting points near or below room temperature. When crystals were obtained, they usually were too soft or were otherwise unsuitable for x-ray analysis. 5-Thiatetradecanoic acid (S-5) was an exception. It crystallized in two distinct conformations, thus allowing us to see some variation in bond length and angles within the same analog.

The two conformations of S-5 are illustrated in Fig. 3 and are designated S5A (top panel) and S5B (bottom panel). It is interesting to note that these conformational differences are largely from position 6 to the carboxyl group (C-1). The C-S distances in S5A are 1.79 and 1.83 Å. This is only slightly different from the 1.75 and 1.82 Å values found in S5B, but "markedly" different from the C-C distances. A greater variation occurs in C-S-C bond angles. These angles are 104.6° and 103° for S5A and S5B, respectively. In S5A, the three-carbon unit from C-2 to C-4 is nearly gauche-gauche. C-3 and C-4 are essentially eclipsed in S5B, although the C-3–C-2 relationship appears similar. Furthermore, as shown in Fig. 3, the carboxyl group of S5A is roughly parallel to the C-4–S bond whereas it is nearly perpendicular in S5B. The structures are similar from the sulfur atom to the terminal methyl groups of each conformer.

Thus, the major structural differences between C, O, or S analogs is that in the latter, one portion of the molecule will have longer bond distances and more acute angles. From the perspective of electronic effects, basicity will increase in the
order C < S < 0. Protonation of O or S under physiological conditions is not expected but hydrogen bond formation, especially with O, is. Furthermore, oxygen will be more strongly solvated in aqueous solution than either C or S (i.e. greater electronegativity leads to extensive hydrogen bonding). As shown in Fig. 2B, elution profiles of analog CoAs from a C8 column confirm the hydrophilicity order C < S < O for the series.

**Analysis of Single Oxygen and Sulfur-substituted Myristic Acid Analogs**—An in vitro survey of the substrate properties of compounds S-3–S-13 and O-3–O-13 was undertaken. An initial reaction was performed using radiolabeled CoA, unlabeled analog, and *Pseudomonas* acyl-CoA synthetase to make certain that the extent of conversion of the various analogs to their acyl-CoA derivatives was comparable. The results indicated that for all single heteroatom-substituted analogs, the efficiency of conversion was similar to myristate, ranging from 50–70% (Fig. 2, A and C, and data not shown).

Incorporation of unlabeled analog into GAR[3H]ASVLS-NH2 derived from the NH2-terminal sequence of a known N-myristoyl protein, the Pr55<sup>+</sup> of HIV-1 (Ratner et al., 1985), was then assessed. Initially, fixed concentrations of analog (15 μM) and peptide (25 μM) and E. coli-derived, purified S. cerevisiae NMT (0.5 μg/ml, 10 μM) were used in a coupled assay described under “Experimental Procedures.” (Table II) shows that the apparent <i>K</i><sub>m</sub> of this peptide in the presence of myristoyl-CoA is 12 μM while the apparent <i>K</i><sub>m</sub> of myristoyl-CoA = 2 μM. The reaction was allowed to proceed for 10 min and the quantity of analog peptide produced compared to the amount of myristoyl peptide generated in a parallel reaction. The results are presented in Fig. 4, A and B, and suggest that heteroatom substitution is well accommodated along the length of the myristate backbone. Our expectation that some dramatic difference would be observed in positions 3 and 4 was not met although both O-3 and S-3 showed modest 3-fold reductions in activity compared to myristate.

Detailed kinetic analyses were conducted on S-3, S-6, S-9, and S-12 as well as the corresponding oxygen analogs (Table II). The apparent <i>K</i><sub>m</sub> of S-12 as well as the corresponding oxygen analogs (Table II) shows that the apparent <i>K</i><sub>m</sub> of this peptide in the presence of myristoyl-CoA is 12 μM while the apparent <i>K</i><sub>m</sub> of myristoyl-CoA = 2 μM. The reaction was allowed to proceed for 10 min and the quantity of analog peptide produced compared to the amount of myristoyl peptide generated in a parallel reaction. The results are presented in Fig. 4, A and B, and suggest that heteroatom substitution is well accommodated along the length of the myristate backbone. Our expectation that some dramatic difference would be observed in positions 3 and 4 was not met although both O-3 and S-3 showed modest 3-fold reductions in activity compared to myristate.

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**Table II**

*Analysis of the interaction of oxygen- and sulfur-containing myristic acid analogs with E. coli-derived S. cerevisiae NMT.*

The apparent <i>K</i><sub>m</sub> of this peptide in the presence of myristoyl-CoA is 12 μM while the apparent <i>K</i><sub>m</sub> of myristoyl-CoA = 2 μM. The reaction was allowed to proceed for 10 min and the quantity of analog peptide produced compared to the amount of myristoyl peptide generated in a parallel reaction. The results are presented in Fig. 4, A and B, and suggest that heteroatom substitution is well accommodated along the length of the myristate backbone. Our expectation that some dramatic difference would be observed in positions 3 and 4 was not met although both O-3 and S-3 showed modest 3-fold reductions in activity compared to myristate.

Detailed kinetic analyses were conducted on S-3, S-6, S-9, and S-12 as well as the corresponding oxygen analogs (Table II). The results suggest that the acyl-CoA binding site is relatively insensitive to the placement of group 6B heteroatoms. Very modest reductions were produced in peptide catalytic efficiency (<i>V</i><sub>max</sub>/<i>K</i><sub>m</sub>) as a result of the binding of some analog CoAs, most notably S-3 and O-3. This can be correlated with the results noted in the single point survey.

*In Vitro Analysis of Analogs with Multiple Heteroatom Substitution*—An obvious question arising from these observations was whether multiple heteroatom substitutions could be as readily tolerated as single group 6B substitutions. Ten compounds were prepared to assess the effect of more than one heteroatom (Fig. 4C). Ease of synthesis was important in our selection of analogs.

Two analogs, O-6,9,12 and O-7,10,13, were very poorly incorporated into acyl-peptide in the single point assay (<5%
of myristate, Fig. 4C). This was true with the HIV-1-derived octapeptide as well as another peptide (GS[^3]H]AASARR-NH₂) whose kinetic properties are similar (in the presence of myristoyl-CoA (Heuckeroth et al., 1990). Under the conditions used, the limited resolution of our HPLC separation system for such hydrophilic analogs-CoA did not allow us to ascertain whether failure to produce acyl peptide reflected the inability of these compounds to serve as substrates for either Pseudomonas CoA synthetase and/or S. cerevisiae NMT.

Removal of one oxygen from either of these two structures (yielding O-6, O-12, O-9, O-12, and O-7, O-10, O-10, O-13, respectively) resulted in some improvement in activity although in each case the amount of acyl peptide produced was 2–20% of that observed with myristate (Fig. 4C). For O-9, O-12, O-7, O-10, and O-10, O-13, this was not due to a failure to form CoA derivative (see Fig. 2C and data not shown). Surprisingly, O-6, O12 was a very poor substrate for the reportedly nonspecific Pseudomonas acyl-CoA synthetase (Shimizu et al., 1980) even though its apparent hydrophobicity was comparable to the other two compounds (i.e. equivalent to hexanoic acid, see Fig. 2B and Fig. 5).

Replacement of one oxygen at either the 6-, 9-, or 12-position of these dioxatetradecanoic acids with sulfur resulted in a complete “rescue” of activity (within experimental error) when compared to myristic acid (see Fig. 4, B and C).

Kinetic analyses were performed to assess the potential mechanisms which yield rescue of activity in di-oxygen-substituted analogs (Table II). We were able to determine peptide Kₘ and Vₘₐₓ for the O-6, O-12 compound by increasing the temperature of the initial acyl-CoA generating step to 37 °C. This had the unexpected effect of increasing the efficiency of analog conversion to the CoA thioester from 10–15% to 70–80% of that of myristate.

Binding of both dioxatetradecanoic acids (O-6, O-12, O-9, O-12) to NMT produce a dramatic change in its peptide binding site, resulting in a 20- and 25-fold decrease in its affinity for GAR[^3]^H]ASVLS-NH₂. In the case of O-6, O-12, this was accompanied by a 20-fold reduction in Vₘₐₓ, an effect which was not noted with O-9, O-12. A 500-fold difference in peptide catalytic efficiency between O-6, O-12 and myristate was observed (Fig. 6) providing a remarkable example of binding site cooperativity in NMT. Replacement of one of the two oxygens in O-6, O-12 and O-9, O-12 with sulfur results in a general increase in peptide catalytic efficiency with values comparable to single heteroatom derivatives (Fig. 6). This suggests that the combination of oxygen and sulfur in the positions surveyed results in compounds that have kinetic properties similar to those analogs containing carbons in the positions occupied by oxygen.

The effects of these replacements on the acyl-CoA-binding site were less dramatic than those observed at NMT’s peptide-binding site (Table II).

These results indicate that a remarkable range of heteroatom substitutions are accommodated by this enzyme which displays extraordinary chain length specificity in vivo. The presence of three oxygen atoms within a single analog apparently exceeds the enzyme’s tolerance for reductions in hydrophobicity and rescue is only partial in the two-oxygen case. Sulfur, a heteroatom differing from carbon more than oxygen does in its bond lengths and bond angles but making less demand in the sense of hydrophobicity, is better accommodated by the enzyme. The dipole moments of carbon-oxygen and carbon-sulfur bonds could also affect positioning within the binding pocket. Furthermore, when two or more heteroatoms are present, dipoles will tend to orient to reduce internal energy and to maximize binding interaction energies. These effects should be greatest in analogs having multiple oxygen substitutions compared to those containing either sulfur or no heteroatoms. This dipolar phenomenon should be strongly position-dependent because the orientation of the dipoles will also be affected by the conformation of the inter-acting carbons. However, if the intramolecular dipolar interactions are “unfavorable,” we would anticipate that they would result in changes in the apparent Kₘ values of these analog-CoAs (compared to myristoyl-CoA). This does not appear to be the case. Functional groups may be available in the enzyme’s active site that are capable of binding to heteroatoms of increased polarity. A resulting change in protein conformation could produce alterations in the peptide-binding pocket. Rescue of dioxatetradecanoic acids by single sulfur substitution would thus be viewed as reflecting the fact that sulfur, a less polar atom that is more like CH₂ than oxygen, does not require the enzyme to undergo the degree of restructuring that is necessary with oxygen and, therefore, fewer

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**Fig. 5.** C₈ reverse-phase HPLC of acylpeptides. Panel A displays the relationship between acyl chain length (C7:0–C16:0 FA were tested) and the retention time of the corresponding enzymatically generated acyl- GARASVLS-NH₂ on the C₈ column. The data are expressed relative to the myristoyl peptide. This acyl peptide elutes with 60–65% buffer B (see “Experimental Procedures” for further details). Note that the difference in retention time in this gradient is comparable to the difference in % buffer B (acetonitrile). Panel B plots the relationship between the retention time (in min) of various analog peptides (peptide = GAR[^3]H]ASVLS-NH₂) and the amount of acyl peptide generated in the in vitro NMT assay. The data on the y axis were taken from Fig. 4, A–C.
Substrate Specificity of N-Myristoyltransferase

TABLE III
Names and structures of myristic acid analogs with aromatic and cyclohexyl residues

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Structure</th>
<th>Compound name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ph-(CH₂)₆-COOH</td>
<td>9-Phenylnonanoic acid</td>
</tr>
<tr>
<td>2</td>
<td>Ph-(CH₂)₇-COOH</td>
<td>10-Phenyldecanoic acid</td>
</tr>
<tr>
<td>3</td>
<td>Ph-(CH₂)₈-COOH</td>
<td>11-Phenylundecanoic acid</td>
</tr>
<tr>
<td>4</td>
<td>CH₃C₆H₄-S-(CH₂)₆-COOH</td>
<td>12-Phenylundecenoic acid</td>
</tr>
<tr>
<td>5</td>
<td>CH₃C₆H₄-(CH₂)₆-COOH</td>
<td>9-(4-Tolyl)nonanoic acid</td>
</tr>
<tr>
<td>6</td>
<td>CH₃C₆H₄-C₆H₅-(CH₂)₆-COOH</td>
<td>8-(4-Phenyl)octanoic acid</td>
</tr>
<tr>
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<td>CH₃C₆H₄-(CH₂)₆-(CH₂)₆-COOH</td>
<td>7-(4-n-Propylphenyl)heptanoic acid</td>
</tr>
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<td>8</td>
<td>CH₃C₆H₄-(CH₂)₆-(CH₂)₆-COOH</td>
<td>6-(4-n-Butylphenyl)hexanoic acid</td>
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<td>9</td>
<td>CH₃C₆H₄-(CH₂)₆-COOH</td>
<td>10-(4-Tolyl)decanoic acid</td>
</tr>
<tr>
<td>10</td>
<td>CH₃C₆H₄-(CH₂)₆-COOH</td>
<td>9-Cyclohexylnonanoic acid</td>
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<tr>
<td>11</td>
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<td>10-Cyclohexylnonanoic acid</td>
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<tr>
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<td>Ph-O-(CH₂)₆-COOH</td>
<td>10-Phenoxynonanoic acid</td>
</tr>
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<td>10-Phenylthioundecanoic acid</td>
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<td>9-(4-Tolylthio)nonanoic acid</td>
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<tr>
<td>16</td>
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<td>9-(4-Phenyl)octanoic acid</td>
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<tr>
<td>17</td>
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<td>9-(4-Methoxynonanoic acid</td>
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<td>9-(4-Methylphenyl)nonanoic acid</td>
</tr>
<tr>
<td>19</td>
<td>CH₃C₆H₄-(CH₂)₆-COOH</td>
<td>12-(2-Fural)decanoic acid</td>
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<tr>
<td>20</td>
<td>CH₃C₆H₄-(CH₂)₆-COOH</td>
<td>12-(2-Thietyl)decanoic acid</td>
</tr>
</tbody>
</table>

![Fig. 6. The peptide catalytic efficiency (%Vₐ₅₀/Kₐ) obtained in the presence of several analog-CoAs with multiple heteroatom substitutions. See Table II and the text for details.](image_url)

conformational changes are “transduced” to NMT’s peptide-binding domain(s).

Analysis of Aromatic Analogs—In a previous study, we noted that S. cerevisiae NMT was able to accommodate the branched chain fatty acids isomyristic acid and anteisopen-tadecanoic acid (Heuckeroth et al., 1990). These results suggested that the acyltransferase could “tolerate” a greater degree of bulk than that presented by myristate. The A value (a measure of free energy conformational difference between axial and equatorial substituted cyclohexanes) is identical for S. cerevisiae NMT was able to accommodate the enzyme. Therefore, two aromatic ring-containing analogs, 11-phenylundecanoic acid and 8Z-11-phenylundecenoic acid, were examined in the same study (Heuckeroth et al., 1990). These were also found to be substrates although their kinetic properties in vitro were poorer than myristate. The Substrate Properties

General Strategy for Design and Synthesis of Analogs—Based on these earlier observations, we decided to systematically study aromatic and heteroaromatic ring systems located at various positions in the fatty acid. Our initial synthetic strategy was guided by two principal questions: (i) Is the optimal chain length of an aromatic-containing analog equivalent to one containing a group 6B heteroatom? (ii) What is the optimal position of the aromatic ring within the chain length defined by (i)? A subordinate question was whether heteroatoms present in an aromatic-substituted analog functioned as expected based on the findings presented above.

Most versatile synthetic approach proved to be the use of the Wittig reaction of aromatic aldehydes with ω-halocarboxylic acids followed by hydrogenation of the resulting olefin:

\[
\text{Ar(CH}_2)_n\text{CH-O+ PhP}^+\text{(CH}_2)_n\text{COOH Br}^- \rightarrow \text{Ar(CH}_2)_n\text{CH=CH(CH}_2)_n\text{COOH + H}_2 \text{Pd-C} \\
\text{Ar(CH}_2)_n\text{CH=CH(CH}_2)_n\text{COOH} \rightarrow \text{Ar(CH}_2)_n\text{CH=CH(CH}_2)_n\text{COOH}
\]

Alternate strategies that were used are described under “Experimental Procedures.” The FA analogs produced in this series are listed in Table III.

The Substrate Properties of ω-Substituted Aromatic Phenyl Analogs of Varying Chain Length—Examination of Corey-Pauling-Koltun (CPK) space-filling atomic models indicates that the width of an aromatic ring is equivalent to three methylene units. The cross-section of the aromatic ring taken perpendicular to the mean plane of the carbon atoms in the ring is 3.4 Å, a value which is approximately equal to the “thickness” of a methylene group. The bonding positions of an aromatic ring are at 60° angles to one another, and all substituents radiate from the aromatic nucleus in the same plane. Unlike the methylene group, the aromatic nucleus is capable of charge transfer interactions using the pi-electrons above and below the carbon plane. Finally, the distance bridged by the para-positions of a benzene ring is equivalent to three methylenes but involves six carbon atoms.

Based on these considerations, we synthesized a series of five FAs from octanoic to dodecanoic having terminal phenyl groups. These were expected to be equivalent in chain length to C-11-C-15 FA. Each of these five compounds are converted to their CoA thioesters with efficiencies that are equal to, or greater than myristate (Table IV). Fig. 7 presents the results of single point surveys of these compounds in parallel NMT assays using three different radiolabeled octapeptides. The best substrate among these compounds had a phenyl ring at the ω end of decanoic acid (10-phenyldecanoic acid). Analogs having one or two more or fewer methylene groups were markedly poorer substrates even though CPK molecular models had suggested that the 11-phenylundecanoic acid...
TABLE IV

Kinetic analysis of aromatic and cyclohexyl analogs of myristate

Details of the coupled in vitro NMT assay using GNAAS[125]YRR-NH₂ were provided in two earlier reports (Heuckeroth et al., 1988b; 1990). Briefly, the apparent peptide Kᵣ and V_max were determined with 15 μM acyl-CoA and varying concentrations of GNAAS[125]YRR (10–80 μM). Plots of [S]/V versus [S] (Hanes-Woolf plots) were linear with a slope of 1/V_max, and a Y intercept of Kᵣ/V_max. Peptide V_max is expressed as a percentage of that obtained with myristate (10 ± 5 pmol/min/μg NMT; n = 47 experiments). Analog-CoA Kᵣ and V_max values were determined with 40 μM GNAAS[125]YRR-NH₂ and varying concentrations of acyl-CoA (0.2–3 μM) for selected compounds and are provided in the text.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AcylCoA formed (nM)</th>
<th>Km (μM)</th>
<th>V_max (%)</th>
<th>ωTime (%)</th>
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<tbody>
<tr>
<td>0-(CH₂)₆-COOH</td>
<td>168</td>
<td>33</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>0-(CH₂)₇-COOH</td>
<td>176</td>
<td>12</td>
<td>210</td>
<td>18</td>
</tr>
<tr>
<td>0-(CH₂)₈-COOH</td>
<td>136</td>
<td>22</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>0-(CH₂)₉-COOH</td>
<td>107</td>
<td>24</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>0-(CH₂)₁₀-COOH</td>
<td>176</td>
<td>12</td>
<td>210</td>
<td>18</td>
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<tr>
<td>0-(CH₂)₁₁-COOH</td>
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<td>8</td>
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<td>157</td>
<td>12</td>
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<tr>
<td>0-(CH₂)₁₆-COOH</td>
<td>168</td>
<td>13</td>
<td>40</td>
<td>1</td>
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<tr>
<td>0-(CH₂)₁₁-COOH</td>
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<td>8</td>
<td>2</td>
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<tr>
<td>0-(CH₂)₁₂-COOH</td>
<td>176</td>
<td>12</td>
<td>210</td>
<td>18</td>
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<tr>
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<td>8</td>
<td>21</td>
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<tr>
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<tr>
<td>CH₃-(CH₂)₂-COOH</td>
<td>160</td>
<td>13</td>
<td>157</td>
<td>12</td>
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<tr>
<td>CH₃-(CH₂)₃-COOH</td>
<td>144</td>
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<td>136</td>
<td>31</td>
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<tr>
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<td>165</td>
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<td>CH₃-(CH₂)₅-COOH</td>
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<tr>
<td>CH₃-(CH₂)₆-COOH</td>
<td>187</td>
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<tr>
<td>CH₃(CH₂)₇-COOH</td>
<td>107</td>
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<td>25</td>
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<tr>
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<tr>
<td>CH₃(CH₂)₉-COOH</td>
<td>178</td>
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<tr>
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<td>100</td>
<td>9.4</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 7. The substrate properties of five fatty acids of differing chain length having ω-terminal phenyl groups.

should represent the FA with the optimal chain length. These relationships were the same for each of the three octapeptides (Fig. 7).

A recent analysis of straight chain fatty acids in the range C-10-C-16 indicated that C13:0 was accommodated nearly as well as C14:0 by S. cerevisiae NMT (Rudnick et al., 1990). C15:0 and C12:0 were poorer substrates than C13:0 but better than C16:0. This led us to conclude that the enzyme's "measuring apparatus" can accommodate a length variation ("error") of one methylene group in straight chain FA. (This is compatible with the fact that odd chain length FAs are very rare in vivo and therefore NMT would not be required to select for, or against, them.) We determined the catalytic efficiencies (V_max/Kᵣ) of the radiolabeled octapeptide GNASAS[125]YRR-NH₂ with each of these five analogs (Table IV). The results indicate that 10-phenyldecanoic acid, when converted to its acyl-CoA derivative produces a 10-fold increase in peptide catalytic efficiency compared to the nonanoic or undecanoic acid derivatives. These differences reflect effects on both apparent Kᵣ and V_max (see Table IV).

With GNASAS[125]YRR-NH₂ at saturating conditions, the Kᵣ of 10-phenyldecanoic-CoA (1.2 μM) is comparable to that of myristoyl-CoA (3.8 ± 2.9 μM, n = 9 experiments). The corresponding V_max of the aromatic analog-CoA is 195% of the V_max obtained with myristoyl-CoA. The acyl-CoA catalytic efficiency of 11-phenylundecanoic acid is also very similar to that of myristoyl-CoA (20 versus 26). Thus, its failure to serve as a good substrate for S. cerevisiae NMT in vitro largely reflects "unfavorable" perturbations produced in the enzyme's peptide binding site (cf. the role of binding site cooperativity in determining the substrate properties of dioxygenated acids discussed above). The Substrate Properties of Fatty Acid Analogs with a Terminal Cyclohexane—The saturated equivalent of a benzene ring is cyclohexane. The van der Waals volumes of cyclohexane and benzene are similar. The orientation of the hydrogen in the 4-position to the substituent in the 1-position (necessarily equatorial for the present cyclohexane derivatives) is also similar. The cyclohexane ring is more hydrophobic by virtue of the smaller carbon to hydrogen ratio. The benzene ring is more polar by virtue of its pi-electron clouds. All the atoms in the benzene ring are planar and resonance stabilized, making this a rigid structure. Cyclohexane, on the other hand, is inherently more flexible even though it occupies a volume similar to that of benzene.

Fig. 8 shows the results of single point surveys of three fatty acids (nonanoic, decanoic, and undecanoic) each with a terminal cyclohexane ring. The relative activities of these analogs parallel those obtained with the comparable phenyl-substituted FA. This suggests that the enzyme's measuring apparatus distinguishes on the basis of bulk rather than polarity or hydrophobicity, at least in this structural series. (Note that no difference was observed in their activities as substrates for Pseudomonas CoA synthetase, see Table IV.)
Detailed kinetic analyses were used to further define the enzyme "responses" to the extent of hydrogenation in the terminal ring of these decanoic acids (Table IV). Curiously, the peptide-binding site was more sensitive in the corresponding octanoic acids than in the nonanoic acids: when compared to the phenyl-substituted species, nonanoic acids had similar $K_a$ and $V_{max}$ values, whereas octanoic acid had similar peptide catalytic efficiency, but an almost 10-fold increase in peptide affinity. Since cyclohexane is a more flexible structure than benzene, this may improve its ability to adjust its structure within the binding pocket and to bind to the enzyme, but not its ability to proceed through the reaction.

Positional Effects of the Phenyl Group within the FA Chain—We further explored the unexpected finding that S. cerevisiae NMT could accommodate a phenyl group in the $\omega$ position of a FA with a second series of experiments. Since 10 carbons plus a phenyl group proved optimal in the previous survey, we prepared a set of derivatives having the same chain length but with the phenyl placed at varying positions within that chain. Because it was difficult to synthesize analogs in which the chain separating the phenyl group from the carboxyl was shorter than five carbons, we focused our attention on the more readily accessible derivatives. Six analogs having an equivalent length of 13 carbon atoms were prepared in which the phenyl group was systematically moved one methylene group closer to carboxyl at a time.

The data presented in Fig. 9 shows that movement of the phenyl group from the $\omega$ end of a 13-carbon equivalent analog one carbon closer to the carboxyl markedly decreases the amount of acyl-peptide produced (for all three octapeptides studied). This was not the result of differences in acyl-CoA production (Table IV). Kinetic analyses indicated that this structural change caused a 3-fold reduction of peptide catalytic efficiency (Table IV) even though the analog-CoA's apparent $K_a$ and $V_{max}$ values were virtually identical to those of 10-phenyldecanoyl-CoA (1.0 versus 1.2 $\mu$M, 11 versus 22%, data not shown).

Another analog allowed us to conclude that this effect on peptide $V_{max}/K_a$ did not arise because a methylene was now present at the FA terminus but because the distance between the phenyl ring and the carboxyl group had been reduced. The 14-carbon equivalent analog 10-(4-toly)decanoic acid has the same relative positions of phenyl and carboxyl as 10-phenyldecanoic acid even though a methyl group is present on the phenyl ring. Its peptide $K_a$ and $V_{max}$ are virtually indistinguishable from 10-phenyldecanoic acid (Table IV).

This remarkable result suggests that the "measuring device" in the enzyme's acyl-CoA-binding site must have two components of sensitivity, distance along the chain from carboxyl to the $\omega$ end and a sensor that detects steric volume at the $\omega$ terminus. These data also suggest that the overall shape of the sensor may be conical with the cone and the terminal carbons co-axial. The balance of sensitivity to length versus steric volume must be determined by the acuteness of the "cone."

Further analysis (Fig. 9) of the effects of progressive movement of the phenyl group using the single point assay raised the possibility that some form of recognition occurs in the central portion of the FA chain: e.g. 7-(4-n-propylphenyl) heptanoic acid is a very poor substrate for NMT while 6-(4-n-butylphenyl) hexanoic acid and 8-(4-ethylphenyl)octanoic acid have activities similar to myristic acid (with all three octapeptides). This alternating trend may be extensible to 9-(p-tolyl)nonanoic acid although some peptide specific differences were encountered (Fig. 9). (Note all four analogs were converted to their CoA thioesters with comparable efficiencies, see Table IV.) These trends defined in the single point assay are paralleled by changes in peptide catalytic efficiency (Table IV).

The Effects of Heteroatom Substitution in Aromatic Analogs—We undertook an additional study to assess whether activity would correlate better with aromatic ring presence and position or with heteroatom presence and position. The group of compounds "available" for these analyses were in the 14 carbon equivalent series. As noted earlier, the HPLC elution times of analog peptides were used to determine that the hydrophilicity of substituted aliphatic analogs increases in the following order: C < S < O (see Fig. 5). The data presented in Fig. 10 show that this relationship is preserved in the presence of an aromatic ring. For example, compare 9-(p-methoxyphenyl)nonanoic acid (compound 17) to 9-(p-ethylphenyl)nonanoic acid (compound 16). The latter is equivalent to tridecanoic acid while the former has the approximate hydrophilicity of undecanoic acid.

Single point surveys of the nine compounds shown in Fig. 11 indicate that the activities of analogs are not affected by oxygen or sulfur substitution or placement in three different aromatic analogs. Their introduction does not override the dramatic effects noted when the position of the phenyl group is altered in the C-14 equivalent FA series.

An interesting feature of the 13 carbon series alluded to above was the "alternating" nature of the activities of the analogs that accompanied one carbon displacements of the phenyl group (Fig. 9). The activity profiles of members of the 14 carbon series shown in Fig. 10 and Table IV could be anticipated on the following basis. Those compounds having a terminal phenyl should not fit into the postulated "conical" receptor and should, therefore, have a lower activity than the three analogs having terminal methyl groups. Since methoxy-, methylthio-, and ethyl- should also be accommodated by this receptor in a similar fashion so long as the aromatic ring is displaced toward the carboxyl, one could predict these three analogs also to have good activity. They do not. Rather, their activities parallel the 13 carbon series, suggesting that the enzyme's measuring apparatus responds to distances from the carboxyl as well as from the terminal end of the FA.

Finally, we synthesized the three terminal aromatic derivatives of dodecanoic acid containing phenyl, 2-furyl, and 2-thienyl to examine whether placement of sulfur or oxygen within the aromatic ring would alter activity. As expected, the thienyl residue was less polar than sp$^2$-hybridized sulfur (see

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4 Other shapes are obviously possible based on the results obtained with this panel of analogs (e.g. parabolic, hemispherical).
FIG. 10. C18 reverse-phase HPLC of aromatic- and cyclohexyl-containing analog peptides. The left hand panel shows a line defining the linear relationship between the length of C-6-C-16 saturated fatty acids \((\bullet)\) and the percent acetonitrile required to elute acylGNAAS-LZ51-YRR-NH2 from the chromatographic matrix. The gradient conditions were as follows: 100% buffer A (0.1% trifluoroacetic acid, 0.05% triethylamine prepared in \(H_2O\)) to 100% buffer B (acetonitrile, 0.1% trifluoroacetic acid) at a rate of 2% B/min. A 3.9 \(\times\) 300-mm Waters \(\mu\)Bondapak C18 column was used. The numbers superimposed upon the line refer to the %B (acetonitrile) needed to elute the same octapeptide containing each of the 23 analogs listed in the right panel. Note that the numbering system used to denote each compound is the same as that used in the miniprint supplement describing their syntheses.

The effects of heteroatom substitution in aromatic analogs.

Thus, the hydrophilicity order in this series was \(C=S>O\).

Single point assays (see Fig. 8) and kinetic analyses (Table IV) indicated little effect of heteroatoms within the aromatic ring on peptide kinetic properties. This may again reflect the aromatic ring dominance in this series.

Together, these studies indicate that NMT possesses a complex measuring device sensitive to FA chain length, bulk, and the placement of steric bulk. The measurement obtained by this device appears to result from triangulation, i.e. the 1- and 14-positions or their equivalent and the location of any substituent along the chain.

Analysis of E, Z, and Y Isomers of Myristic Acid—Our functional analyses of myristic acid analogs in which one or more methylene was replaced by oxygen, sulfur, and/or an aromatic ring indicated that \(S.\ cerevisiae\) NMT exhibits surprising selectivity related to heteroatom identity and placement as well as aromatic subunit placement. These studies also revealed potential differences between nonbonding electron pairs (heteroatom series) versus pi-bonding (aromatic series). Myristic acid derivatives containing double and triple bonds also offer the possibility of examining positional effects and differences in pi bond interactions. In addition, the unsaturated units permit subtle, and sometimes great conformational variation to be introduced throughout the molecule by systematic synthesis of three analog series (trans \((E)\) double bonds, cis \((Z)\) double bonds, and triple bonds). Earlier studies of four analogs (Z-9-tetradecanoic acid (myristoleic acid, Z9), E-9-tetradecanoic acid (myristelaidic acid, E9), 11-phenylundecanoic acid, and 8Z-11-phenylundecenoic acid) indicated that the presence of a double bond and its placement could affect the NMT's peptide-binding site (see Heuckeroth et al., 1988b; 1990).

We, therefore, synthesized 12 myristic acid analogs con-
various standards, we determined that the purity of the E6 chemically. Based on gas chromatographic analysis using were synthesized. We were able to obtain pure Z isomers required to achieve this degree of purity (see Miniprint). cis-double bond-containing analogs (positions 3 through 13), containing triple bonds beginning at positions 2 through 13, 11 triple bond analogs have a four-carbon subunit within each structure that is required to be coaxial. When the Y and corresponding E analogs are compared using CPK models, they appear to be structurally similar but the volume occupied by the Y isomer appears to be larger because of the extended "kink." The basicities with respect to protonation or hydrogen bond formation should be similar for all mono-unsaturated analogs. The pi directionality will be less defined in the Y isomers than in either the corresponding E or Z isomer. This means that protonation, for example, could occur from any direction in an alkyne but must occur from above or below in an E or Z alkene. The overall length of the alkyne analogs will reflect the short C-C bond distances found in triple bonds (1.21 Å versus 1.54 Å in ethane). This shortening will also occur, but to a lesser extent, in double bond isomers (C=C bond = 1.33 Å). The triple bond system has a colinear element within it (i.e. bond angles = 180°) and the double bond isomers involve sp²-hybridized carbons so carbon-carbon bond angles will be 120° rather than the 109.5° expected for saturated carbon.

Evaluation of Alkynyl Analogs—All possible positional isomers of myristic acid containing a single triple bond replacing one C–C single bond were surveyed in the single point NMT assay. The results obtained with the HIV-1 Pr55Gp6 complex polypeptide precursor-derived octapeptide are shown in Fig. 12A. Several interesting differences were observed. A small but reproducible decrease in acylpeptide production occurred when the triple bond was present in the 8-, 10-, or 11-position of myristic acid. When the triple bond was placed at position 2 or 3, an even greater decrease in product formation was noted. This is analogous to results obtained with group 6B heteroatom-substituted derivatives. The most striking observation is that one compound, 5-tetradecynoic acid (Y5), is not a substrate for NMT (although it is an excellent substrate for the Pseudomonas CoA synthetase included in the reaction mixture, see Fig. 13). This failure to generate detectable levels of acyl peptide (i.e. <1% of myristoyl peptide) was not a feature specific for the HIV-1-derived peptide, a similar result was obtained with GS[H]AASARR-NH₂ (data not shown). Y5-CoA failed to block the incorporation of [³H]myristoyl-CoA into the two octapeptide substrates over a broad concentration range (up to a 100-fold molar excess, data not shown), suggesting that this analog does not bind to S. cerevisiae NMT.

Evaluation of Alkenyl Analogs of Myristic Acid—Tetradecenoid acids having double bonds from positions 3 through 13 were synthesized. We were able to obtain pure Z isomers (>98% Z as judged by gas chromatography and/or by NMR) in most cases. In some instances, special purification was required to achieve this degree of purity (see Miniprint). Three E isomers and the terminal double bond isomer (no stereochemistry, D13) were also prepared and characterized chemically. Based on gas chromatographic analysis using various standards, we determined that the purity of the E6 and E7 isomers was greater than 99%. E5 appeared to be greater than 99% pure as assessed by gas chromatography, i.e. we could readily detect 5% isomer contamination in an E6/Z6 mixture and could detect none in the E5 sample. [¹³C] NMR at 100 mHz showed major peaks at 128.97 and 130.58.
ppm corresponding to the double bond carbons of E5. Each peak was accompanied by a much smaller peak offset slightly (<0.1 ppm) from it. If these minor peaks represent the Z isomer double bond carbons, if the nuclear Overhauser enhancements are identical for E5 and Z5, and if the linewidths are identical, then the NMR study indicates that the purity of the E5 isomer preparation is >93%. Since no Z isomers was detected by gas chromatographic analysis, we believe 7% isomer contamination is an overestimate.

The results of single point analysis of the Z series of myristic acid analog are presented in Fig. 12B. The amount of acyl-GARASVLS-NH₂ produced after addition of D₁₃, Z₁₂-Z₅ was similar and comparable to that obtained with myristate. This contrasts with the results noted for the Y series. In the alkene series, Z₃ and Z₄ showed quite low activity as did Y₂ and Y₃ in the alkyne series. The greatest contrast in activities was noted between Y₅, which was not a substrate, and 2₅ which was superior to all other double bond isomers. Interestingly, the E₅ isomer was inferior to E₆ and E₇, both of which were comparable to myristate in acyl peptide formation (see Fig. 12C).

An unexpected observation was made after introduction of Z₆ into the NMT assay system. This analog consistently yielded two acyl-peptide products that were resolvable by CIS reverse-phase HPLC (see Fig. 12B and 14). These two acyl peptide peaks were designated A and B. Only single peaks of radioactivity were noted under comparable conditions for Z₅ and Z₇ (Fig. 14). This phenomenon was reproduced with the other tritiated octapeptide, GS[³⁵S]AASARR-NH₂ (data not shown). When the E₆ isomer was introduced into the NMT reaction mixture, a single peak, corresponding to radiolabeled acyl peptide was observed (see right column of Fig. 14). When equal amounts of the E₆ and Z₆ isomers were included in the reaction mixture, both peak A and peak B were observed but the height of B increased relative to A (see Fig. 14). Addition of Y₆ to the NMT assay system generated a single peak of radioactivity with a retention time different from either E₆ or Z₆ (Fig. 14). Together these data suggested that peaks A and B correspond to Z₆ and E₆, respectively, and that the Z analog is isomerized by an as yet undefined mechanism during the course of the reaction. An important control experiment was performed to determine whether the generation of peaks A and B was related to the activity of NMT or the Pseudomonas acyl-CoA synthetase. This involved addition of Z₆, E₆, or Y₆ to a mixture of the synthetase and [³H]CoA. The resulting radiolabeled acyl-CoAs were purified by reverse-phase HPLC as described in the preceding papers. Z₆ and E₆ produced single acyl-CoA derivatives which could be resolved by HPLC on a C₁₈ column (see Fig. 14). Thus, production of peaks A and B appears to occur after binding of Z₆-CoA to NMT.

An alternative interpretation of how peaks A and B are generated from Z₆ (other than isomerization) is that the kinetic properties of E₆ are vastly superior to those of Z₆ and that a minor degree of contamination of the Z₆ preparation permits selective binding or reaction of one isomer. This explanation seems unlikely for several reasons. First, the single point assay (Fig. 12) and kinetic analyses using two peptides suggest that E₆ and E₇ have similar acyl-CoA and peptide catalytic efficiencies (see Tables V and VI plus Fig. 15). These values differ little from those calculated for the corresponding Z isomers. Second, a time course study was also performed in which 15 µM Z₆ and 1.5 µM E₆ were incubated with the Pseudomonas CoA synthetase, NMT, and [³H]GARASVLS-NH₂. The ratio of peak A to peak B did not change significantly over a 20-min period (Fig. 16).

Conclusions—The data presented here permit us to speculate.

**TABLE V**

Kinetic characterization of unsaturated fatty acid analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>GARASVLS-NH₂</th>
<th>Acyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵣ, µM</td>
<td>Vₑₓₕ, % of Myr</td>
</tr>
<tr>
<td>Myristate</td>
<td>12 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>Z₇</td>
<td>19</td>
<td>65</td>
</tr>
<tr>
<td>Z₆</td>
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<td>138</td>
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<tr>
<td>Z₅</td>
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<td>E₇</td>
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<td>106</td>
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<td>ND</td>
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<td>134</td>
</tr>
<tr>
<td>Y₅</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**FIG. 14.** HPLC analyses of the reaction products generated after addition of Z₆, E₆, and Y₆ to the Pseudomonas acyl-CoA synthetase assay containing [³H]CoA (left panel) or to the coupled S. cerevisiae NMT reaction mixture containing GAR[³H]ASVLS-NH₂.
late about the nature of *S. cerevisiae* NMT’s acyl-CoA-binding site. Based on recent work (Rudnick et al., 1990, 1991), it appears that (i) the enzyme’s active site must possess one or more recognition points for the CoA moiety of acyl-CoA and

![Fig. 15](image-url)  
**Fig. 15.** Catalytic efficiencies of two NMT octapeptide substrates in the presence of Z, E, and Y isomers. Data plotted in this figure was obtained from kinetic analyses described in Tables V and VI.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GSAASARR-NH₂</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>% of Myr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristate</td>
<td>12 ± 3</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z₁</td>
<td>26</td>
<td>39</td>
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<td></td>
</tr>
<tr>
<td>Z₆</td>
<td>19</td>
<td>197</td>
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<td></td>
</tr>
<tr>
<td>Z₅</td>
<td>12</td>
<td>85</td>
<td></td>
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</tr>
<tr>
<td>E₇</td>
<td>16</td>
<td>68</td>
<td></td>
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<td>E₆</td>
<td>28</td>
<td>120</td>
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<td></td>
</tr>
<tr>
<td>E₅</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₇</td>
<td>17</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₆</td>
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<td></td>
</tr>
<tr>
<td>Y₅</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 16](image-url)  
**Fig. 16.** Time course of production of peaks A and B after addition of either the Z₆ or E isomer preparations to the coupled *in vitro* NMT assay. Incubations were done in parallel. Aliquots of the reaction mixtures (containing GAR[¹³H]ASVLS-NH₂) were withdrawn at the indicated times and subjected to C₁₇ reverse-phase HPLC (see Fig. 14). The size of peak A and B was determined by integration of the area under the radiolabeled peak (accomplished using the software package that is included in the Radiomatic Detector).

![Fig. 17](image-url)  
**Fig. 17.** Hypothetical schematic representation of the conformation of the acyl chain of myristoyl-CoA in the acyl-CoA binding site of *S. cerevisiae* NMT. Bond angles are approximate. See text for discussion.

(ii) it is able to interact with acyl-CoA in the absence of peptide.

Previous high resolution x-ray studies performed in our laboratory indicated that palmitate bound to a small intracellular cytosolic protein synthesized in intestinal enterocytes (intestinal fatty acid binding protein) exhibits a distinctive bent conformation from the carboxylate group to C-16 (Sachetti et al., 1989). The bent methyleane chain resides in a cradle formed by the side chains of a number of hydrophobic, principally aromatic, amino acids. For example, a major bend occurs in the fatty acid between C-1 and C-4. These carbons are within van der Waals contact distance of a Trp and Arg residue which stabilize the complex.

We propose that the acyl chain of myristoyl-CoA is also present in a bent conformation in the binding site of NMT. This is shown schematically in Fig. 17. The key features of the model are (i) that the major bend occurs between C-4–C-7, (ii) the first four or five carbons of the fatty acid chain are in an extended conformation, and (iii) the ω end of the FA contacts a conical sensing device that detects both length and bulk. An additional feature is the possible close apposition of a carboxyl side chain of NMT to the C-6–C-7 domain of the bound FA. This putative carboxyl has no obvious role in binding myristate and may well only be revealed by conformational changes induced by binding of FA analogs (e.g. Z₆).

Evidence supporting this model is as follows. The alkyne analog Y₅ in which the linear triple bond would have to reside at the proposed bend is not a substrate. Z₅, on the other hand, is naturally predisposed to this bend because of its double bond geometry and is the most active member of this isomer series. If this structural model is correct, we can account for isomerization in Z₆ because of its positioning at the bend and the proximity of the putative carboxyl group of NMT (Fig. 17). The fact that 5-(4-n-pentylphenyl)pentanoic acid is a good substrate for NMT suggest that the bend is not acute or at least has some flexibility. Finally, the geometry of the terminal sensing device has been at least functionally defined by the effect of adding a terminal methyl group to 10-phenyldecanoic acid. Syntheses of additional analogs that will test this model further are currently underway as are x-ray studies of the protein itself.

**Acknowledgments**—We are indebted to Luis Glaser, Robert Heuckeroth, and Philip Needleman for their continued interest, insights, and encouragement. We thank John D. Ellison for technical assistance.

**REFERENCES**

Experimental Section

General Information: Melting points were determined using a Laboratory Dover MEL-TEMP apparatus, in open capillaries and are uncorrected. NMR: NMR spectra were recorded on a Hitachi Perkin-Elmer R-400 high resolution NMR spectrometer or on a Varian VXR 400 superconducting NMR with 10,000 50000 resonance in CCl_4. IR spectra were recorded in a Perkin-Elmer FT infrared spectrophotometer or on a Perkin-Elmer 200 infrared spectrophotometer. Mass spectra were performed on a Microlab Inc. FT infrared spectrophotometer. Column chromatography was carried out on Merck Kieselgel 60, 230-400 mesh. Low resolution fast atom bombardment (FAB) mass spectra were obtained on a Microlab Inc. UV spectra were obtained on a Shimadzu UV-2100 spectrophotometer. Gas chromatographic analyses were performed on a Shimadzu GC-9A gas chromatograph. The abbreviations define double bond geometry. All chemical reactions were carried out under an atmosphere of nitrogen or argon.

3-Octadecanol Acid, OS

Hydrolysis of the ester (60.0 mg, 0.18 mmol) in ethanol (4.0 mL) was added to the mixture and refluxed for 1.5 h. The mixture was stirred for 0.5 h at room temperature. The mixture was slowly added to water (25 mL) and stirred for 3 h at 55-60 °C. After stirring for 3 h at room temperature, the mixture was added to the mixture and refluxed for 1.5 h. The organic phase was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C.

Synthesis of 3-Octadecanol Acid, OA

Hydrolysis of the ester (60.0 mg, 0.18 mmol) in acetone (25 mL) and 50 mL of water were added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C.

5-Octadecanol Acid, OA

Hydrolysis of the ester (60.0 mg, 0.18 mmol) in acetone (25 mL) and 50 mL of water were added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C.

Synthesis of 8-Octadecanoic Acid, OA

Hydrolysis of the ester (60.0 mg, 0.18 mmol) in acetone (25 mL) and 50 mL of water were added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C.

8-Octadecanoic Acid, OA

8-Octadecanoic Acid, OA

Hydrolysis of the ester (60.0 mg, 0.18 mmol) in acetone (25 mL) and 50 mL of water were added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C. The mixture was added to the mixture and the reaction mixture was incubated at 10 °C and 100 °C.
9-Octadecenoic Acid, 06
Sodium (0.3 g. 13 mmol) was added to distilled 1-pentanol (100 mL) and refluxed for 2 h. Bromoacetone (0.057 g, 0.3 mmol) was added and the mixture was refluxed for 2 days. The reaction mixture was reduced in vacuo, the residue was dissolved in ethyl acetate (200 mL), and the mixture was refluxed for 6 h. The residue was dissolved in ethyl acetate (200 mL), separated by column chromatography on Kugelrohr afforded product (2.15 g, 88% yield; bp 80-85°C/0.05 Torr). IR (KBr): 2930 and 2890 cm⁻¹. 'H-NMR: 0.93 (t, 3H), 1.40 (m, 6H), 2.36 (t, 2H), 2.53 (q, 2H), 2.59 (t, 2H), 3.25 (12H). 

Synthesis of 1-Octadecanol-11-acetic acid

1-Octadecanone (0.71 g. 3.0 mmol) was added and the mixture was refluxed for 8 h. The mixture was reduced in vacuo, and the residue was dissolved in ethyl acetate (200 mL). The mixture was reduced for 6 h. The residue was dissolved in ethyl acetate (200 mL), separated by column chromatography on Kugelrohr afforded product (2.15 g, 88% yield; bp 80-85°C/0.05 Torr). IR (KBr): 2930 and 2890 cm⁻¹. 'H-NMR: 0.93 (t, 3H), 1.40 (m, 6H), 2.36 (t, 2H), 2.53 (q, 2H), 2.59 (t, 2H), 3.25 (12H). 

5-Thiactetradecanoic Acid, 05
Sodium hydroxide (1.0 M, 22 mL) was added to a solution of 5-thiactetradecanoic acid (2.0 g, 7.3 mmol) in MeOH (25 mL). After stirring for 7 h, the reaction mixture was washed with 10% HCl (pH-1) and extracted with ethyl acetate (2.0 mL). The organic phase was washed with water, brine, and dried over Na2SO4. The crude product was synthesized from hexane to afford 88 (1.75 g, 99%; mp 45-45.5°C. IR (KBr): 2900 and 1700 cm⁻¹. 'H-NMR: 0.84 (3H, t, J=4.6 Hz). 1.2-1.4 (14H, m), 1.5-1.6 (6H, m), 2.48 (2H, t, J=7.6 Hz), 4.48 (4H, q, J=7.6 Hz). 7.5-7.6 Hz. and 10.5 ppm (1H, br. s). Analysis: calculated for C21H39NO2, C 33.7; H 6.04%; found: C 33.3; H 5.6%. 

Synthesis of 5-Thiactetradecanoic Acid, 06

Ethyl 5-Thiactetradecanoate: This compound was synthesized from 5-thiactetradecanoic acid (2.0 g, 7.3 mmol) in MeOH (25 mL). After stirring for 7 h, the reaction mixture was washed with 10% HCl (pH-1) and extracted with ethyl acetate (2.0 mL). The organic phase was washed with water, brine, and dried over Na2SO4. The crude product was synthesized from hexane to afford 88 (1.75 g, 99%; mp 45-45.5°C. IR (KBr): 2900 and 1700 cm⁻¹. 'H-NMR: 0.84 (3H, t, J=4.6 Hz). 1.2-1.4 (14H, m), 1.5-1.6 (6H, m), 2.48 (2H, t, J=7.6 Hz), 4.48 (4H, q, J=7.6 Hz). 7.5-7.6 Hz. and 10.5 ppm (1H, br. s). Analysis: calculated for C21H39NO2, C 33.7; H 6.04%; found: C 33.3; H 5.6%. 

Synthesis of 5-Thiactetradecanoic Acid, 08

8-Thio-8-octatomide, 04
Ethyl 8-octatomide: This compound was synthesized from 5-thiactetradecanoic acid (2.0 g, 7.3 mmol) in MeOH (25 mL). After stirring for 7 h, the reaction mixture was washed with 10% HCl (pH-1) and extracted with ethyl acetate (2.0 mL). The organic phase was washed with water, brine, and dried over Na2SO4. The crude product was synthesized from hexane to afford 88 (1.75 g, 99%; mp 45-45.5°C. IR (KBr): 2900 and 1700 cm⁻¹. 'H-NMR: 0.84 (3H, t, J=4.6 Hz). 1.2-1.4 (14H, m), 1.5-1.6 (6H, m), 2.48 (2H, t, J=7.6 Hz), 4.48 (4H, q, J=7.6 Hz). 7.5-7.6 Hz. and 10.5 ppm (1H, br. s). Analysis: calculated for C21H39NO2, C 33.7; H 6.04%; found: C 33.3; H 5.6%.
The synthesis of 12-thiododecanoic acid was carried out from sodium hydride (1.1 g, 46.5 mmol) and hexanal (0.4 mL) in dry THF (10 mL) and the mixture was stirred for 1 h. The crude product was purified by column chromatography (silica gel, hexane/ethyl acetate 1:1). The yield was 75%. To a solution of 5-bromo-12-octadecanethiol (1.6 g, 3.5 mmol) in THF (10 mL), sodium hydride (0.32 g, 60% suspension in oil) was added and the mixture was stirred for 30 min. 1,1-Dimethylhydrazine (0.38 mL) was added and the mixture was stirred for 1 h. The crude product was purified by column chromatography (silica gel, hexane/ethyl acetate 1:1). The yield was 60%. The reaction mixture was poured into water (150 mL) and extracted with diethyl ether (4×50 mL), the organic phase was washed with water, dried over MgSO₄, and the solvent was evaporated. The residue was dissolved in THF (100 mL) and the solution was filtered through a basic alumina column (activated at 110 °C). The yield was 85%. The reaction mixture was poured into water (150 mL) and extracted with diethyl ether (4×50 mL), the organic phase was washed with water, dried over MgSO₄, and the solvent was evaporated. The residue was dissolved in THF (100 mL) and the solution was filtered through a basic alumina column (activated at 110 °C). The yield was 85%. The reaction mixture was poured into water (150 mL) and extracted with diethyl ether (4×50 mL), the organic phase was washed with water, dried over MgSO₄, and the solvent was evaporated. The residue was dissolved in THF (100 mL) and the solution was filtered through a basic alumina column (activated at 110 °C). The yield was 85%.
on silica gel using ethyl acetate/hexane (9:1) as solvent and then distilled (Kugelrohr) to give ethyl 9-phenyl-10-undecenoate (41 mmol in method A) or ethyl 9-phenyl-11-decenoate (41 mmol in method B) or ethyl 9-phenyl-12-decenoate (41 mmol in method C) or ethyl 9-phenyl-13-undecenoate (41 mmol in method D).

Synthesis of 9-Phenyl-12-decanol acid, 90.813
2-Phenyldecan-1-ol (racemic) (12 mmol) in THF (20 mL) was dissolved with potassium hydroxide (12 mmol in 44 mL of THF-H2O (70:30) under nitrogen while stirring for 30 min. The solution was cooled to 0°C and added dropwise to 20% (10 mL) in THF (20 mL) was added dropwise and the mixture was stirred at room temperature for 12 h. The mixture was poured into water (150 mL) and acidified with 2N HCl. Extracted with ethyl acetate (3x200 mL), the organic phase was washed with water, brine, and dried over MgSO4. The crude product was isolated by vacuum distillation (Kugelrohr) to afford 90.813 in 84% yield. IR: 3400-2500, 1715 cm⁻¹; 1H-NMR: 1.29 (2H), 1.35 (2H), 1.48 (4H), 2.30 (4H), 2.85 (4H), 3.70 (3H), 3.96 (3H), 4.75 (4H).

Hydrolysis of 9-Phenyl-12-decanol acid to Give 90.916. A solution of the above compound (8.8 mmol) in 30% NaOH (10 mL) was refluxed for 6 h, the solution was acidified with HCl (pH 4) and extracted with ethyl acetate (120 mL), the organic layer was washed with water, brine, and dried over MgSO4. The substrate was heated in an oil bath at 180-200°C for 20 min. Distillation (Kugelrohr) afforded 90.916 in 98% yield. IR: 3225-2500, 1715 cm⁻¹; 1H-NMR: 1.30 (6H), 1.80 (4H), 2.50 (4H), 3.50 (4H), 3.90 (4H), 4.80 (4H), 4.90 (4H).

General Procedure for Hydrolysis. The unsaturated compound, (Z)-4-fluorocinnamic acid (19.71 g) was added to 20% (10 mL) in THF (20 mL) under nitrogen while stirring for 30 min. The solution was cooled to 0°C and added dropwise to 20% (10 mL) in THF (20 mL) was added dropwise and the mixture was stirred at room temperature for 12 h. The mixture was poured into water (150 mL) and acidified with 2N HCl. Extracted with ethyl acetate (3x200 mL), the organic phase was washed with water, brine, and dried over MgSO4. The solvent was removed in vacuo. Chromatography (silica gel; 1:1 hexane-ethyl acetate) and distillation (Kugelrohr) afforded 90.916 in 98% yield.

9-Phenyl-12-decanol acid was prepared as described in method A from 9-phenyl-11-decenoic acid (8.8 mmol) and HCl (P10:1) in water (20 mL). The solution was acidified (pH 4) and extracted with ethyl acetate (3x200 mL). The solvent was removed in vacuo and the residue was crystallized from petroleum ether or hexane to yield product.
Synthesis of 9-(4-Tolylthio)octadecylphosphonic Acid.

A solution of ethyl 9-(4-tolyloxy)nonanoate (1.40 g, 9.01 mmol) was dissolved in 10 mL of THF (100 mL). Distillation (Kugelrohr) and crystallization (benzene) gave 14.90 g, 87%.

89%.

Synthesis of 9-(4-Tolyloxy)nonanoic Acid.

A solution of ethyl 9-(4-tolyloxy)nonanoate (1.40 g, 9.01 mmol) was dissolved in 10 mL of THF (100 mL). Distillation (Kugelrohr) and crystallization (benzene) gave 14.90 g, 87%.

89%.

Synthesis of 9-(4-Tolyloxy)octadecylphosphonic Acid.

A solution of ethyl 9-(4-tolyloxy)nonanoate (1.40 g, 9.01 mmol) was dissolved in 10 mL of THF (100 mL). Distillation (Kugelrohr) and crystallization (benzene) gave 14.90 g, 87%.
Syntheses of 9-[(2-phenylmorpholino)phenyl]acetic Acid, 18.
9-[(2-Phenylmorpholino)phenyl]acetic acid was prepared as described in method A from 7-carboxy-2-[2-(naphthyl)phenyl]boronic acid (0.75 g, 10 mmol) and triphenylphosphine (1.04 g, 4.0 mmol) in THF (100 mL). Crystallization (pentane/ethanol) gave the unsaturated acid (2.19 g, 80%) as white crystals (mp 48-50°C). IR: 3400-2500, 1735 cm⁻¹.

Sodium formate (0.75 g, 11 mmol) was added to 7-bromo-2-[2-(naphthyl)phenyl]boronic acid (1.40 g, 3.5 mmol) in acetic acid (15 mL) at room temperature. After stirring for 3 d, the mixture was diluted with water, and the solid was filtered and washed with ethanol. The physical and spectral properties compared favorably with those reported to the authors by Dr. M. Misra in 1981 and Gunimza et al. 1977. The isomer ratio was determined by gas chromatography to be 2.9 ± 0.9.

65. Tetradecenoic Acid, 86.
This compound was prepared by dissolving metal reduction according to the procedure detailed below for 73.3 g 2,4-dimethylphosphine (27.7 g, 50 mmol) and n-hexanal (8.40 g, 50 mmol) in THF (100 mL). After 4 h, the mixture was diluted with water, and the solid was filtered and washed with ethanol. The physical and spectral properties compared favorably with those reported in Koga et al. 1974 and Gunimza et al. 1977. The isomer ratio was determined by gas chromatography to be 2.9 ± 0.9.

66. Tetradecenoic Acid, 86.
This compound was prepared by the method described above for 74.3 g 2,4-dimethylphosphine (27.7 g, 50 mmol) and n-hexanal (8.40 g, 50 mmol). After workup and distillation (Kugelrohr, bp 160-163°C/0.1 Torr), the distillate was diluted with water, and the solid was filtered and washed with ethanol. The physical and spectral properties compared favorably with those reported in Koga et al. 1974 and Gunimza et al. 1977. The isomer ratio was determined by gas chromatography to be 2.9 ± 0.9.

67. Tetradecenoic Acid, 87.
This compound was prepared by the method described above for 75.3 g 2,4-dimethylphosphine (27.7 g, 50 mmol) and n-hexanal (8.40 g, 50 mmol). After workup and distillation (Kugelrohr, bp 160-163°C/0.1 Torr), the distillate was diluted with water, and the solid was filtered and washed with ethanol. The physical and spectral properties compared favorably with those reported in Koga et al. 1974 and Gunimza et al. 1977. The isomer ratio was determined by gas chromatography to be 2.9 ± 0.9.

68. Tetradecenoic Acid, 86.
This compound was prepared by the method described above for 76.3 g 2,4-dimethylphosphine (27.7 g, 50 mmol) and n-hexanal (8.40 g, 50 mmol). After workup and distillation (Kugelrohr, bp 160-163°C/0.1 Torr), the distillate was diluted with water, and the solid was filtered and washed with ethanol. The physical and spectral properties compared favorably with those reported in Koga et al. 1974 and Gunimza et al. 1977. The isomer ratio was determined by gas chromatography to be 2.9 ± 0.9.
125-Tetradecynoic Acid, 212

125-Tetradecynoic acid was synthesized by the procedure described above for 5-hexynoic acid. The compound was obtained as a colorless oil.

4-Tetradecynoic Acid, 4

5-[4-(4-Bromophenyl)butyl]cytosine 379 and 8-fluoromethyl 379 were prepared as described in the synthesis of 5-[4-(4-bromophenyl)butyl]-8-fluorocytosine 379 and 5-(4-bromophenyl)cytosine 379. The compounds were obtained as colorless oils.

Synthesis of 5-Tetradecynoic Acid, 5

5-Tetradecynoic acid was synthesized by the procedure described above for 5-hexynoic acid. The compound was obtained as a colorless oil.