Dynamics of a Hydrophobic Peptide in Membrane Bilayers by Solid-State Nuclear Magnetic Resonance†

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Received June 3, 1985

ABSTRACT: Solid-state NMR studies of the dynamics of a synthetic hydrophobic peptide, *tert*-butyloxycarbonylleucylphenylalanine methyl ester (Boc-Leu-Phe-OMe), in phospholipid bilayers are described. Motionally averaged powder pattern line shapes from ^{2}H- and ^{15}N-labeled backbone and side-chain sites of the peptide in phospholipid bilayers demonstrate the presence of both overall and internal reorientations of substantial amplitude. The peptide motions are shown to be strongly influenced by the motions of the lipids.

Many peptides and proteins interact with cell membranes in biological systems. It is of interest to study models of these complex systems in order to be able to describe in detail the physical nature of their inter- and intramolecular interactions. The structure and dynamics of the components of a wide variety of model membrane systems have been studied by physical methods, especially NMR spectroscopy. Applications of solid-state NMR in these studies have been particularly successful in characterizing the motions of phospholipids in bilayers, where the averaging of spin interactions manifested in powder pattern line shapes is indicative of the amplitudes and rates of motions at individual sites. For reviews of NMR studies of phospholipids in bilayers, see Seelig (1977), Jacobs & Oldfield (1981), Griffin (1981), and Davis (1983).

In general, the non-phospholipid components of membranes have been studied less than the phospholipids, in large part because of technical limitations of NMR and other physical methods. Several solution NMR studies of biological peptides have been carried out with phospholipid vesicles and detergent micelles instead of phospholipid bilayers as model membranes (Braun et al., 1983; Brown et al., 1982; Feigenson & Meers, 1980; Wakamatsu et al., 1983; Weinstein et al., 1980). By a combination of isotopic labeling and solid-state NMR methods, a few studies of membrane-bound proteins have provided valuable information about their dynamics. The intramolecular motions of bacteriorhodopsin in purple membrane preparations (Kinsey et al., 1981; Rice et al., 1981; Keniry et al., 1984; Smith & Oldfield, 1984) and the coat protein of the filamentous bacteriophages in phospholipid membrane bilayers (Frey et al., 1983; Bogusky et al., 1985; Coluago et al., 1985) have been characterized in this way. Both of these membrane-bound proteins have polypeptide backbones with most of the sites immobile on the slowest NMR time scales available (10^3 Hz). These proteins differ from globular cytoplasmic proteins in that a substantial number of the backbone sites have rapid large-amplitude motions. The mobile sites appear to be restricted to those portions of the peptide backbone that extend beyond the bilayer into the solvent and head group region. For the most part, the side-chain dynamics of these proteins appear to be similar to those observed for crystalline peptides and proteins. The residues attached to the rigid portion of the protein backbone have one or more rapid jump motions, while those in mobile backbone sites have additional modes of motion. A single ^{2}H NMR spectrum of a simplified model system of a membrane-spanning synthetic peptide gives some indications of its dynamics in bilayers (Callaghan et al., 1984). The large size, complexity, and heterogeneity of proteins in membranes all contribute to the difficulty of describing the dynamics of all of the sites in detail.

Boc-Leu-Phe-OMe is a small hydrophobic peptide that was synthesized because it was expected to interact with the hydrocarbon portion of the phospholipids in membrane bilayers. Studies of this peptide in membrane environments are complementary to on-going studies of proteins and larger peptides in membrane environments. A model membrane system using this peptide has the advantages that a readily synthesized molecule is used, so that isotopic labels can be placed at essentially any location, structural analogues are available, and large amounts of highly purified material can be obtained for the spectroscopy experiments. Figure 1 contains the chemical structure of this peptide with all of the various isotopically labeled sites indicated, although in practice individually labeled peptides were used for the experiments. This peptide presents both aromatic and aliphatic side chains as well as the peptide backbone sites for study.

This model peptide undergoes restricted anisotropic reorientation within the membrane bilayers. Consequently, the line shapes of the motionally averaged powder patterns from isotopically labeled sites reflect the motions of the peptide in the bilayers. Most of the results presented are from ^{2}H NMR, where the quadrupolar interaction dominates the spectra. A few results utilizing the chemical shift anisotropy and dipolar interactions of ^{15}N-labeled peptide groups are also described. In this approach, the presence of motions is indicated by their averaging effects on the powder patterns from the spin interactions; for reviews, see Torchia (1984) and Opella (1985).

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine (DPL) and L-α-dimyristoylphosphatidylcholine (DML) were obtained from Sigma Chemical Co. Deuterium-depleted water was obtained from Aldrich Chemical Co. Isotopically labeled amino acids were from Cambridge Isotope Laboratories and Merck Iso-

† This research was supported in part by a grant from the NIH (GM 29754). M.H.F. was supported by a Cell and Molecular Biology training grant. L.M.G. is a Fellow of the A. P. Sloan Foundation (1984–1986).

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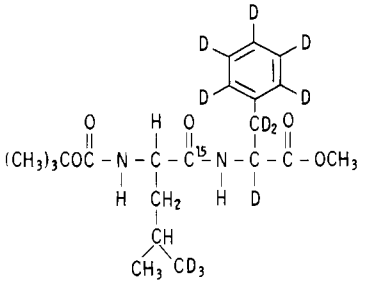

FIGURE 1: Structural formula of Boc-Leu-Phe-OMe showing the various isotopically labeled sites.

topes. The peptides were synthesized by standard methods. Peptide couplings were made by the mixed-anhydride method (Bodansky et al., 1976), using *N*-methylmorpholine and isobutyl chloroformate. The N-terminus of the isotopically labeled leucine was blocked by reaction with *tert*-butyloxycarbonate (Moroder et al., 1975). The methyl ester of phenylalanine was formed by refluxing the amino acid in methanol with $SOCl_2$ (Boissonas et al., 1956). The dipeptide containing L-α-deuterated phenylalanine was synthesized from a racemic mixture of the labeled phenylalanine and then purified by fractional crystallization. Optical purity was checked by circular dichroism. All samples of the dipeptide were crystallized from ethyl acetate/hexane (mp 82–83 °C) and were homogeneous by thin-layer chromatography (R_f 0.69; 97% $CHCl_3$/3% methanol).

Samples for the NMR experiments consisted of peptides in phospholipid bilayers in excess water. The peptides and lipids were mixed in the desired proportions and dissolved in chloroform. The solvent was then removed under vacuum. Subsequently, an excess of deuterium-depleted water was added, and the sample was thoroughly mixed on a vortex stirrer while the temperature was raised and lowered through the gel to liquid-crystalline phase transition several times. Finally, the mixtures were concentrated by centrifugation in an Eppendorf Model 5414 microfuge. The samples were then transferred to a glass container and sealed.

Differential scanning calorimetry was carried out on a Perkin-Elmer DSC-2 calorimeter. The samples were 7–10 mg, and the scanning rate was 2.5 °C/min.

Most of the NMR experiments were carried out on a modified JEOL GX-400 WB spectrometer with a 9.4-T magnet using home-built probes. The data in Figure 8 were obtained on a home-built spectrometer with a 5.7-T magnet. The deuterium NMR probe used a solenoidal coil that had 90° pulse widths of approximately 4 μs under the normal experimental conditions. All deuterium NMR spectra were obtained by the quadrupole echo pulse sequence (Davis et al., 1976) with a typical time interval between pulses of 31 μs and a recycle time of 300 ms. All data were obtained by using quadrature detection with the spectra in Figure 4 and the spectra in Figure 5, except for those from the α site of Phe, presented in symmetrized form.

RESULTS

Boc-Leu-Phe-OMe synthesized with ^2H and ^{15}N in various locations (Figure 1) was incorporated into bilayers made from dimyristoyllecithin (DML) and dipalmitoyllecithin (DPL). Samples with these two similar phospholipids were compared so that the effects of temperature on peptide and lipid dynamics could be separated. Overall, the peptide appeared to behave very similarly in both lipids at temperatures reflecting the differences in the gel to liquid-crystalline transition temperatures of the lipids.

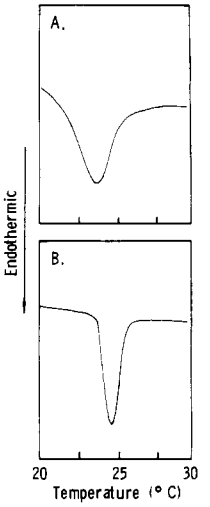

FIGURE 2: Differential scanning calorimetry data obtained with a scanning rate of 2.5 °C/min: (A) Boc-Leu-Phe-OMe in DML (1:15) in excess water; (B) DML alone in excess water.

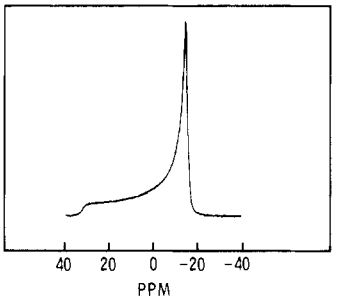

FIGURE 3: ^{31}P NMR spectrum of Boc-Leu-Phe-OMe in DML (1:15) in excess water at 25 °C.

Differential scanning calorimetry (DSC) showed that the peptide effects the phospholipid phase transition. This is illustrated in Figure 2, where the phospholipids containing the peptide (Figure 2A) undergo a thermal transition at a slightly lower temperature that is markedly broadened compared to that of the pure phospholipids in excess water (Figure 2B). Although not shown in the expanded plots of Figure 2, the lipid pretransition disappears completely in the presence of peptide. ^{31}P NMR spectra of the peptide-containing lipid bilayers above and below the lipid phase transition are essentially indistinguishable from those obtained for the lipid alone. The ^{31}P NMR spectrum in Figure 3 of the lipids in the presence of the peptide is characteristic of lipid bilayers and indicates that the peptide does not disrupt the lipid organization to a significant extent. In addition, the longitudinal relaxation time (T_1) for the phosphorus resonance is not altered by the presence of the peptide. Taken together, these control experiments show no evidence of the peptide inducing nonbilayer structures in the membranes and suggest that the peptide interacts largely with the hydrocarbon chains of the phospholipids.

Most of the findings in this paper are derived from ^2H NMR spectra of specifically labeled peptides. Peptides with deuterium atoms on the methyl groups of the Leu residue and on the α, β, and ring sites of the Phe residue were studied. Before describing the spectroscopic results in detail, it is important to note that at high peptide to lipid ratios there is strong evidence of peptide aggregation in some of the samples. Complex multicomponent peptide NMR spectra are observed when large amounts of peptide are incorporated into the phospholipid bilayers. Saturation of the concentration-de-

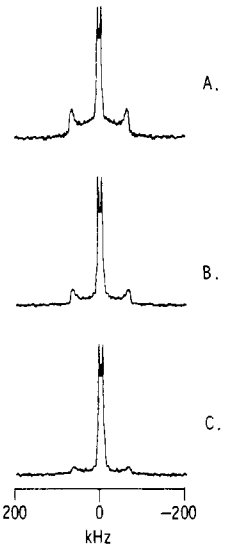

FIGURE 4: ^2H NMR spectra of [$^2H_5$]Phe labeled Boc-Leu-Phe-OMe in DML in excess water at 25 °C at various peptide:lipid ratios: (A) 1:2; (B) 1:7.3; (C) 1:15.

pendent broadening and lowering of the DSC transition are also observed. Figure 4 demonstrates the effect of peptide concentration on the ^2H NMR spectrum of the Phe-labeled peptide. Note the presence of two spectral components, one of which is an essentially static powder pattern that we ascribe to peptide aggregates that form at high peptide to lipid ratios and the other of which is a motionally narrowed powder pattern due to peptide molecules within the bilayer environment. The relative amount of the static spectral component increases with increasing amount of peptide. In analyzing the relative concentrations of the mobile and immobile peptide side chains, it must be kept in mind that the static pattern is spread out over a much larger frequency breadth than the motionally averaged one. Although these data are complicated by Phe having multiple labeled sites on a side chain that can readily undergo internal motions, they do indicate that these experiments reflect the phase separations and transitions in these systems. In order to avoid complications from this separation effect, all of the experiments on the temperature dependence of the line shapes were done on samples with peptide to lipid ratios of 1:15.

^2H NMR spectra of the labeled peptides in lipids were obtained over a wide temperature range. Spectra obtained at −120, 10, and 60 °C of deuterium-labeled peptides in DML bilayers are shown in Figure 5. The spectra from all of the labeled sites exhibit a strong temperature dependence. At −120 °C, the deuterium powder patterns are essentially the same as expected from a rigid lattice and observed for rigid crystalline peptides; hence, the large-amplitude rapid motions present at higher temperatures are frozen out at low temperatures. Drastic narrowing of the powder patterns from all sites occurs at moderate and high temperature. Not only are the spectra narrowed at higher temperatures, but the line shapes are also very different than observed for the static case and change with temperature. There are distinct differences in line shapes between 10 and 60 °C. All of these spectral parameters indicate a large extent of motional narrowing; however, even at the highest temperatures studied the narrowing is not complete since no isotropic line shapes were observed. Above the gel to liquid-crystalline phase transition, the line shapes of the ^2H NMR powder patterns are constant, and there is a gradual narrowing of the entire pattern with increasing temperature.

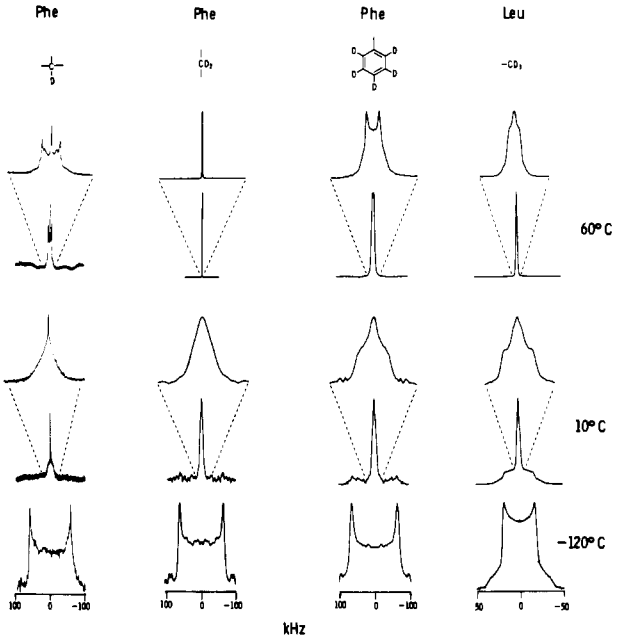

FIGURE 5: ^2H NMR spectra of Boc-Leu-Phe-OMe labeled at the designated sites with deuterium in DML (1:15) in excess water. All spectra were obtained by the quadrupole echo pulse sequence with an interpulse time of 31 μs and a recycle delay of 300 ms.

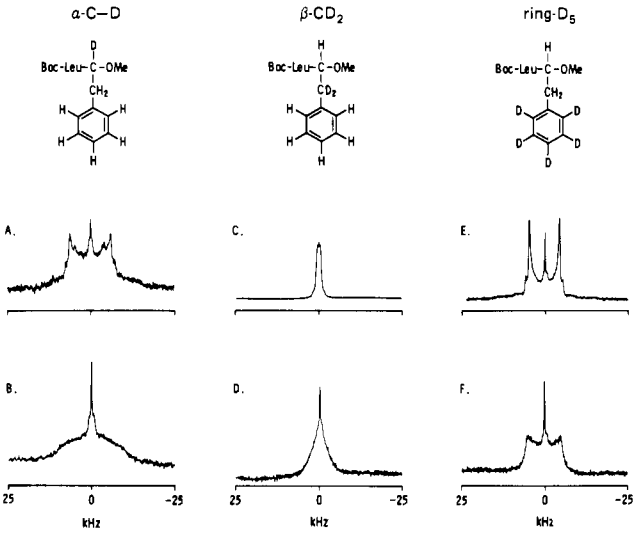

FIGURE 6: ^2H NMR spectra of Boc-Leu-Phe-OMe in DML (1:15) in excess water. Spectra A, C, and E are at 25 °C, slightly above the DML phase transition temperature, and spectra B, D, and F are at 20 °C, slightly below the DML phase transition temperature.

One of the main aspects of peptide–lipid interactions that we wanted to address with this model system concerns the possible effects of lipid motions on peptide motions. Lipid motions strongly influence peptide motions as demonstrated by the data in Figures 6 and 7. The spectra in these figures were obtained in a narrow temperature range around the lipid phase transition temperatures for both DML and DPL bilayers containing Boc-Leu-Phe-OMe. The spectra in Figure 6 are at 25 and 20 °C, which are just above and just below the phase transition temperature of DML alone. There are clearly drastic differences in line shapes of Phe-labeled peptide sites caused by the lipid phase transition. The methyl-labeled Leu residues display different behavior, without such large changes around the lipid phase transition temperature. The spectra obtained of the same labeled sites in DPL at 50 and 38 °C on both sides of its transition temperature, are shown in Figure 7 and are similar to those observed for DML near its phase

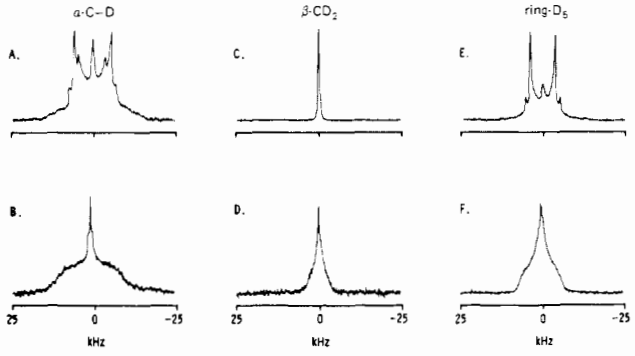

FIGURE 7: ^2H NMR spectra of Boc-Leu-Phe-OMe in DPL (1:15) in excess water. Spectra A, C, and E are at 50 °C, slightly above the DPL phase transition temperature, and spectra B, D, and F are at 38 °C, slightly below the DPL phase transition temperature.

transition temperature, differing only in the shape of the ring resonances in Figures 6F and 7F. However, the ring line shapes are actually very similar at a few degrees lower temperature as can be seen by comparing the 10 °C spectrum in Figure 5 (DML) to that in Figure 7F (DPL). None of these line shapes are well simulated by the kinds of spectra that are characteristic of one or several well-defined jump motions. Therefore, an analysis of rapid rotational or jump reorientation about single bonds on an immobile backbone is not appropriate. Unusual line shapes and intensities can result from simple motions at "intermediate" exchange rates. If the spectra in figures reflected the effects of motions with "intermediate" time scales, then the observed line shapes would be strongly influenced by the experimental parameters of the quadrupole echo pulse sequence, especially the interpulse spacing during which very efficient relaxation can occur. However, the line shapes and intensities in the figures are relatively insensitive to the length of delay between the two pulses in the echo sequence in the range of 30–100 μs.

^{15}N NMR spectra were obtained for backbone-labeled peptide in DPL bilayers. If the backbone were fully immobilized by the peptide–lipid interactions, then a chemical shift powder pattern of approximately 170 ppm breadth would be observed in the presence of high-power proton decoupling. Instead, a narrow (10 ppm) resonance was observed in the spectrum in Figure 8A, indicating that the peptide backbone undergoes extensive motional averaging on the 10^3-Hz time scale of the chemical shift interaction. This is consistent with the findings from the ^2H NMR spectra in Figure 5 from the α-labeled site. However, proton decoupling does provide some narrowing of the resonance as seen in the comparison of the spectra in Figure 8, indicating that the motional averaging is incomplete on the time scales of the heteronuclear dipolar interaction (10^4 Hz). The ^{15}N–^1H NOE was found to be negative for these samples, indicating that rapid motions near the Larmor frequencies (10^9 Hz) are present as well.

Discussion

The synthetic hydrophobic peptide Boc-Leu-Phe-OMe interacts with the hydrocarbon chains of the phospholipids in model membrane bilayers. The phase transition temperature of the phospholipids is lowered slightly and is broadened substantially in the presence of the peptide. The lipid pretransition disappears in the presence of the peptide. The ^{31}P NMR spectra of the lipids show essentially no change in the presence of peptides. These results strongly indicate that while the peptide is influenced by lipids and the lipids are influenced by the peptide, the basic bilayer structure is not disrupted by these interactions. The peptide dynamics are complex and vary

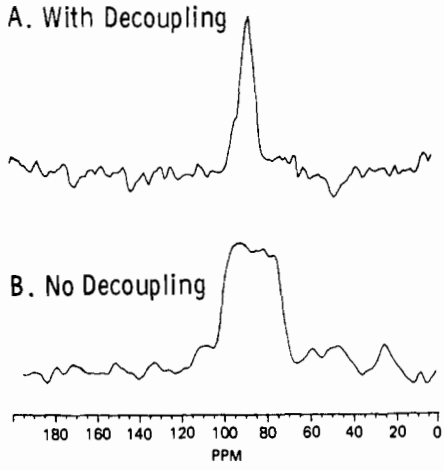

FIGURE 8: ^{15}N NMR spectra of Boc-Leu-Phe-OMe in DML (1:15) at 25 °C: (A) with proton irradiation; (B) without proton irradiation.

with temperature and the ratio of the numbers of peptide molecules to lipid molecules in the sample.

At high peptide to lipid ratios (greater than 1 to 15), there is clear evidence that peptide aggregation occurs by both DSC and NMR. The temperature dependence of the separation of the peptides into mobile and immobile phases indicates that these NMR experiments can be useful in describing phase diagrams of mixed-lipid systems.

The collection of ^2H and ^{15}N NMR data on various labeled sites on the peptide described under Results cannot be fully interpreted with simple models of peptide dynamics. However, several qualitative conclusions emerge from these data. One of the clearest is that the peptide dynamics are strongly influenced by lipid dynamics as seen in the large differences in spectra from peptide sites at temperatures a few degrees above and below the lipid phase transition. The motional averaging reflected in NMR data from labeled peptide sites can appear to be complex from several possible influences: (1) the simultaneous presence of "fast" and "intermediate" frequency motions for some or all of the peptide sites; (2) relatively rapid interconversion among peptides in several states with different dynamics, such as monomers and aggregates; (3) a combination of well-defined rapid motions that involve overall reorientation of peptide molecules within the bilayers and jump or diffusional motions about individual bonds in the peptide. Of course combinations of these and other possibilities may occur. Case 1 can be ruled out by ^2H NMR experiments in which the interpulse spacing was varied, since there was little change of line shape as a function of pulse interval. If there were intermediate frequency motions on the ^2H NMR time scale (100 kHz), then these line shapes should be highly sensitive to this parameter. It is difficult to rule out possibility 2, and at high peptide to lipid ratios evidence of peptide aggregates was observed. However, those aggregates had immobile sites, and the molecules interchanged slowly between the two populations. Case 3 is undoubtedly true even if the others are as well.

The range of ^2H NMR spectra cannot be explained by the peptide having either of the extreme cases: a rigid backbone or an isotropically reorienting backbone. Both ^2H and ^{15}N backbone labeled sites show extensive but incomplete motional narrowing of the resonance lines. The peptide itself must be undergoing anisotropic reorientation within the lipid bilayers. Motions of individual groups in the peptide also occur because of the variety of line shapes observed in the spectra; however, it is the motion of the peptide itself that must explain the greater extent of motional narrowing of the β-deuterium sites

than the ring sites. The angle of peptide reorientation relative to the membrane bilayer must be such that the angle for the β-deuterons is fairly close to the magic angle in order to account for the extremely narrow lines. The β-deuteron line shapes contrast greatly with those from the adjacent α or side-chain sites.

The motional properties of Boc-Leu-Phe-OMe are quite different from those observed for larger polypeptides in bilayer environments. The filamentous bacteriophage coat proteins (Frey et al., 1983; Bogusky et al., 1985; Coluago et al., 1985), bacteriorhodopsin (Keniry et al., 1984), and a synthetic polyleucine peptide (Callaghan et al., 1984) all have most of their peptide backbone sites immobilized on the NMR time scales in model membranes, with only those parts extending beyond the hydrocarbon region into the solvent with substantial mobility. Both hydrocarbons (Jacobs et al., 1984; de Ropp et al., 1984) and lipids (Taylor et al., 1982) in model phospholipid bilayers have motional properties that appear to be at least superficially similar to those observed for Boc-Leu-Phe-OMe in bilayers, with complex combinations of overall and intramolecular reorientations. Previous work with small hydrophobic peptides in micellar environments (Gierasch et al., 1982, 1983) and vesicles (Jain et al., 1985) suggests that these peptides reside in the interfacial region of the hydrocarbon chains near the head groups, resulting in mobility that is greater than that found in polypeptides that are influenced by the full length of the hydrocarbon chains.

Registry No. DPL, 63-89-8; DML, 18194-24-6; Boc-Leu-Phe-OMe, 5874-73-7; Boc-Leu-Phe-OMe (labeled), 99560-61-9.

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