Barley Lipid Transfer Protein, LTP1, Contains a New Type of Lipid-like Post-translational Modification*

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In plants a group of proteins termed nonspecific lipid transfer proteins are found. These proteins bind and catalyze transfer of lipids in vitro, but their in vivo function is unknown. They have been suggested to be involved in different aspects of plant physiology and cell biology, including the formation of cutin and involvement in stress and pathogen responses, but there is yet no direct demonstration of an in vivo function. We have found and characterized a novel post-translational modification of the barley nonspecific lipid transfer protein, LTP1. The protein-modification bond is of a new type in which an aspartic acid in LTP1 is bound to the modification through what most likely is an ester bond. The chemical structure of the modification has been characterized by means of two-dimensional homonuclear nuclear magnetic resonance spectroscopy as well as mass spectrometry and is found to be lipid-like in nature. The modification does not resemble any standard lipid post-translational modification but is similar to a compound with known antimicrobial activity.

A group of proteins termed nonspecific lipid transfer proteins (ns-LTPs)1 is found in plants. Originally these proteins were identified by their ability to catalyze the transfer of lipids between membranes in vitro (1). The suggestion that they would act as intracellular transporters of lipids between organelles was later questioned because of the fact that ns-LTPs are extracellular proteins. Other functions have been ascribed to ns-LTPs, including transport of cutin monomers (2) and involvement in flowering (3). Also ns-LTPs have been suggested to be important in several types of plant stress response. These include responses to pathogens (4), drought (5), and temperature changes (6, 7). Despite their implication in these diverse aspects of plant biology, it is not clear which specific role ns-LTPs play here (8, 9).

ns-LTPs are small proteins of about 90 residues with high values of pI (>9). The three-dimensional structures of ns-LTPs from several plant species are known, and they all consist of four α helices held together by four conserved disulfide bonds (10). A hydrophobic central cavity is found between the four helices. In vitro studies have shown that ns-LTPs can bind a variety of fatty acids (11) and lipids (12). High-resolution structures of ns-LTPs in complex with fatty acids are available from barley (13) and maize (14). In these structures the fatty acid occupies the central hydrophobic cavity. However, it has been difficult to draw any conclusions about the in vivo activity of ns-LTPs from their lipid binding properties because it is unknown which ligands, if any, are bound to ns-LTPs in vivo.

ns-LTPs are encoded by multigene families (15). In a given plant, several ns-LTP genes can be found, and they are often specifically expressed in both time and tissue. Also individual ns-LTP genes are induced under a variety of conditions. It is not clear whether the various ns-LTPs have functional overlap. If this is the case, it may prove difficult to use genetic approaches in determining the function of ns-LTPs.

This paper describes experiments on an aleurone-specific barley ns-LTP, LTP1 (16, 17). The LTP1 protein is abundant in the barley grain, from which it can be purified. The results show that most of this LTP1 is modified post-translationally by a new type of modification. As the modification is lipid-like in nature, this is the first direct demonstration that an ns-LTP binds lipids in vivo.

EXPERIMENTAL PROCEDURES

Materials—Barley grains (cv optic) were purchased from Dansk Landbrugs Grovvareelskab. Trypsin (TPCK-treated, sequencing grade) was from Promega. 2-vinylpyridine was from Aldrich. Tris(2-carboxyethyl)phosphine was from Sigma.

LTP1 Purification—Approximately 0.5 kg of barley grains was ground, and the resulting flour was extracted in water overnight at 4 °C at pH 6.5. After centrifugation, (NH4)2SO4 was added to 40% saturation, and the pellet was discarded. Subsequently (NH4)2SO4 was added to 75% saturation. Following precipitation, the pellet was resuspended in water and dialyzed overnight against water. The dialyzed solution was centrifuged, and the pH in the supernatant was adjusted to 6.5. This solution was loaded onto a large CM-52 carboxymethyl cellulose column (2.6-cm internal diameter, 30 cm long) equilibrated with 20 mM potassium phosphate buffer, pH 6.5 (Buffer A). After a stable baseline was obtained, proteins were eluted with a linear gradient using 0.25 liters of Buffer A and 0.25 liters of Buffer B (Buffer A + 0.25 M KCl). Fractions were pooled and dialyzed overnight against water. Then the pH was adjusted to 7.0, and the sample was loaded onto an SP-Sepharose column (1.6-
Post-translational Modification of Barley LTP1

Barley LTP1 was purified by a method similar to that published previously (27). Initial MS studies of the purified protein revealed two peaks, one centered at the mass expected from the LTP1 sequence (9687 Da, taking the four disulfide bonds into account) and one peak corresponding to a mass ~300 Da higher (Fig. 1). This was a surprise because neither sequencing of the protein (17) nor NMR studies (10) had suggested the presence of a post-translational modification or co-purification of a ligand. The two forms were separated by RP HPLC, and subsequent N-terminal sequencing and amino acid analysis verified whether the modification could simply be a glycosylation. A colorimetric determination of carbohydrates was carried out as described earlier (19). Glucose was used as a standard.

Kinetic Parameters for the Conversion of ptm-LTP1 to um-LTP1—LTP1 was incubated in 150 μl at 2 mg/ml in 0.1 M buffer (potassium citrate, pH 3.1, potassium acetate, pH 4.8, potassium phosphate, pH 7.0, and Tris-HCl, pH 5.2 and pH 9.0, or CAPS, pH 10.0) in sealed glass vials in the autosampler of the HPLC system at 37 °C. At various time points, 20 μl was withdrawn and analyzed for amounts of ptm-LTP1 and um-LTP1 superimpose.

Tryptic Digestion of LTP1—LTP1 was reduced and pyridine-ethylated by dissolving 75 nmol of LTP1 and 52 μmol of the disulfide reductant Tris(2-carboxyethyl)phosphine in 100 μl of 6 M guanidine hydrochloride and 0.1 M potassium phosphate, pH 6.5. After incubation for 30 min at 25 °C, 10 μl of neat 2-vinylpyridine was added, and the mixture was incubated for further 90 min in the dark. After desalting on a NAP-5 column (Amersham Pharmacia Biotech) equilibrated with 20% (v/v) acetic acid, pyridine-ethylated LTP1 was further purified by RP HPLC using a gradient from 0 to 65% acetonitrile (0.1% v/v trifluoroacetic acid) over 60 min (1 ml/min) separated the two forms of LTP1. Protein was detected using online absorption (280 nm) and fluorescence (λexc = 275 nm, λem = 303 nm) measurements. Peak integration allowed determination of the relative amounts of the two forms because the UV absorbance spectra of um-LTP1 and ptm-LTP1 superimpose.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and automatic Edman sequencing. Acid-catalyzed release of the modification from the tryptic peptide was carried out by incubation in 3.8 M HCl for 1 h at 65 °C.

NMR Spectroscopy—ptm-LTP1 was dissolved in 600 μl of 90/10% H2O/D2O to a concentration of 3 mM. All spectra on this sample were recorded on a Bruker AMX600 spectrometer at 14.1 Telsa with a 5-mm triple-resonance probe. The sample temperature was 310 K, and the pH was 7.2. A series of standard two-dimensional homonuclear spectra were recorded on this sample, including DQF-COSY (20), TOCSY (21), and NOESY (22). Also a heteronuclear natural abundance 13C-HSQC spectrum was collected (23).

The N-terminal tryptic peptide from ptm-LTP1 was purified as described above and dissolved in 40 μl of MeOH-D2O to an approximate concentration of 8 mM. All spectra were recorded on a Varian Unity spectrometer at 11.7 Telsa with a 4-mm 1H NMR nanoprobe. The sample temperature was 298 K, and the spinning rate was ~2 kHz at the magic angle (45° ± 5°) with respect to the broad line. Standard homonuclear experiments were recorded, including DQF-COSY, TOCSY (24), NOESY, and ROESY (25). Additionally, natural abundance 13C-HMQC and 13C-HMB-C (26) spectra were collected.

Mass Spectrometry—Low resolution MALDI MS (mass spectrometry) was carried out on a Finnigan Lasermat 2000 time-of-flight spectrometer using α-cyano-4-hydroxycinnamic acid as matrix. Nano-electrospray ionization mass spectra were acquired on a Q-TOF mass spectrometer (Micromass). The cone voltage was adjusted to the lowest possible at which complete declustering of the analyte was obtained, typically 30–35 V. The peptides were desalted on a nanoscale C18 column prior to analysis. Appropriate ions were selected in the first quadrupole and fragmented in the hexapole collision cell with a collision energy of ~40 eV. Argon was used as collision gas at a pressure of 6 × 10–5 millibar. The fragment ions were detected in the TOF-reflector analyzer at a mass resolution of ~5000. Spectra in negative ion mode were obtained under the same conditions as those in positive mode by switching all voltage polarities.

RESULTS

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The number of possibilities, tryptic peptides were generated by the sequence the modification is bound. To narrow down the number, the analysis was to localize to which amino acid in the LTP1 reaction by nonlinear regression. Obtained by the integration of HPLC peaks to a pseudo first-order reaction catalyzed. However, for a simple hydroxide-catalyzed reaction, one would expect a straight line with slope of 1 in a log ($k_{obs}$) versus pH plot (28). However, in Fig. 2C, the slope is around 0.4, indicating that the reaction is not a simple hydroxide-catalyzed hydrolysis. Nevertheless, this type of pH profile is indicative of an ester or an amide bond, the first being the more base-labile.

Identification of the Modified Amino Acid—The next step in the analysis was to localize to which amino acid in the LTP1 sequence the modification is bound. To narrow down the number of possibilities, tryptic peptides were generated by the digestion of denatured, reduced, and vinylpyridine-treated pmt-LTP1. The reduction and alklylation of LTP1 were carried out near neutral pH to minimize base-catalyzed hydrolysis of the protein-modification bond. The isolation of individual tryptic peptides by RP HPLC and analysis by MS and sequencing showed that the modification was bound to the N-terminal tryptic peptide constituted by residues 1–9: Leu-Asn-Cys-Gly-Gln-Val-Asp-Ser-Lys. This modified peptide eluted much later than and well separated from the other tryptic peptides, suggesting that the modification is hydrophobic in nature. The high-resolution electrospray mass spectrum of the fraction containing the modified peptide showed doubly and triply charged ions $m/z$ 681.93 and 454.97, respectively, corresponding to a peptide with a molecular mass of 1361.9. In addition, a very minor doubly charged peak at $m/z$ 534.8 indicated the presence of the unmodified peptide (molecular mass of 1067.6, calculated mass of 1067.52). The presence of small amounts of unmodified peptide in the sample was presumably caused by the hydrolysis of the peptide-modification bond during sample handling. These results show that the additional mass attributable to the modification is 294.3 ± 0.1. Full-length Edman sequencing of the peptide showed the presence of all nine amino acids except the cysteine in position 3. However, because the cysteine thiol in pmt-LTP1 is accessible to modification with 2-vinylpyridine, this residue is very unlikely to be the site of modification. The data in Fig. 2 show that the hydrolysis of the protein-modification bond is base-catalyzed. Presumably the alkaline conditions of the coupling reaction in the Edman degradation caused at least partial hydrolysis of the protein-modification bond, thereby making it impossible to determine the modified amino acid by Edman sequencing. It was therefore decided to attempt sequencing by tandem mass spectrometry because this technique is well established for the site-specific assignment of post-translational modifications in peptides and proteins (29). The selection and collision-induced dissociation of the doubly charged ions at $m/z$ 681.9 and 534.8 showed a nearly complete series of $y$ and $b$ ions (30) for the modified as well as for the unmodified peptide (Fig. 3 and Table I). Based on abundant $y_2$ and $y_3$ ions in the MS/MS spectra of both compounds, the modified amino acid residue is unambiguously identified to be aspartic acid 7. The selection and dissociation of the triply charged ion at $m/z$ 455, although resulting in a slightly more complex spectrum because of additional charge state fragment ions, confirm that Asp-7 is the modified amino acid residue (data not shown).

Analysis of the Chemical Structure of the Modification—An obvious next step was to determine the chemical structure of the modification. Negative results in the phenol sulfuric acid test ruled out most types of glycosylation. Furthermore, UV absorbance spectra of pmt-LTP1 and um-LTP1 could be superimposed, excluding strong chromophores in the modification. A comparison of two-dimensional homonuclear spectra of um-LTP1 and pmt-LTP1 revealed signals that could only arise from the modification (data not shown). Because LTP1 is an all-helix protein, the chemical shifts from a carbon carbon double bond could easily be distinguished from protein-derived signals. By measurement of the H–H coupling constant, the double bond was found to be in a cis configuration. Most other signals originating from the modification were found in the crowded aliphatic region of the spectra. Thus, it was not possible to determine the chemical structure of the modification from these spectra.

Because the protein-modification bond is more base-labile than peptide bonds (Fig. 2) and Asp-7 is the modified residue (Fig. 3), it is likely that an ester bond connects the modification to the protein. We have been able to cleave this bond at least
partially by both hydroxylaminolysis (31) and methanolysis (32). However, difficulties with detection of the free modification have hampered this approach. It was therefore decided to use the N-terminal tryptic peptide as a "handle" for studying the modification further.

Using nanoprobe two-dimensional 1H NMR, it was possible to assign all protein-derived protons in the peptide. This allowed for the identification of signals originating from protons in the modification. The intensities of these signals were comparable with those originating from the peptide. Fig. 4 shows part of a two-dimensional TOCSY spectrum together with a plausible assignment. The high field shifted signal is indicative of an omega methyl group in an aliphatic carbon chain. Noteworthy is the carbonyl in the suggested structure which terminates the spin system. On the basis of DQF-COSY and TOCSY spectra, several spin systems were observed, and it was possible at least partially to assign all of these (Fig. 5). Fig. 5 also shows a structure which is compliant with all our NMR and MS data. To confirm the suggested structure, we unsuccessfully collected spectra that rely on through-space transfer of magnetization (NOESY and ROESY). However, further information was obtained by the collection of natural abundance 13C-HSQC and 13C-HMBC spectra. These spectra verified the structures suggested from the homonuclear experiments. The HSQC spectrum shows one-bond hydrogen carbon correlations, giving a fingerprint of each hydrogen-bound carbon in the structure. In contrast the HMBC spectrum, which arises from two- and three-bond hydrogen carbon correlations, was helpful in assigning nonprotonated carbon nuclei. As an example Fig. 6 shows the NMR evidence for the presence of a carboxylic acid in the modification using a combination of homo- and heteronuclear experiments.

Attempts to obtain information about the structure of the prosthetic group by positive ion MS3 of the tryptic peptide in an

![MS/MS spectra](image)

**Fig. 3.** MS/MS spectra after selection of the doubly charged ions (labeled P) corresponding to the modified peptide (m/z 681.9) (A) and the unmodified peptide (m/z 534.7) (B). A series of y ions, which unambiguously identify the modification to Asp-7, are labeled. For clarity, decimals are omitted on the mass assignment of the peaks. For details on all observed sequence ions, refer to Table I.

| Table I | Expected and observed sequence ions for the peptide LNCGQVD*SK |
|--------|------------------------------------------------------------|
| b ion  | Modified Unmodified Modified Unmodified                   | y' ion |
|        | Amino acid residue | 114.0 114.0 | LEU | 1249.8* | 955.4 |
|        |                   | 228.2 228.2 | ASN | 1135.8* | 841.4 |
|        |                   | 436.3 436.3 | CYS | 927.7*  | 633.3 |
|        |                   | 493.3 493.3 | GLY | 870.6*  | 576.3 |
|        |                   | 621.4 621.4 | GLN | 742.6*  | 448.2 |
|        |                   | 720.5 720.5 | VAL | 643.4*  | 349.2 |
|        |                   | 1129.8 835.5 | ASP | 234.1  | 234.1 |
|        |                   | 1216.8 922.5 | SER | 147.1  | 147.1 |

The observed ions are indicated in bold. The asterix indicates sequence ions containing the modified amino acid residue.
electrospray ion trap mass spectrometer were not successful because no ion corresponding to the modifying group could be located in the MS2 spectrum. However, acid-catalyzed hydrolysis of the peptide-modification bond allowed for analysis of the free modification by MS. Fig. 7 shows electrospray ionization spectra in both positive and negative ion mode of a sample obtained by incubating modified peptide in 3 M HCl at 65°C. Desalting was omitted prior to mass spectrometric analysis of this sample because passage over the C18 column might cause loss of the liberated modifying group. In positive ion mode, we observed a peak at m/z 335.2 corresponding to a sodium adduct of the free modification. Attempts to obtain further information about the structure of this ion by MS/MS did not result in any fragmentation. In negative ion mode, a peak corresponding to the deprotonated form of the modification was observed at m/z 311.5. In addition, this spectrum contains a series of peaks caused by sodium chloride clusters, (NaCl)\(_n\), n = 3–8. These account for the groups of peaks centered around m/z 211, 269, 329, 387, 445, and 503 and are easily recognized because of the multiple chlorine isotope patterns. The selection and collision-induced dissociation of the ion at m/z 311.5 gives rise to the spectrum shown in Fig. 7C. This spectrum is dominated by successive losses of water and carbon dioxide as reported to be characteristic for low energy collision-induced dissociation of monohydroxylated unsaturated fatty acid anions (33). In addition, numerous low abundance fragment ions derived from the cleavage of the hydrocarbon backbone are observed, many of which are not visible in Fig. 7C. Nearly all fragment ions can be rationalized based on the proposed structure, assuming that hydrogen rearrangement reactions take place as observed in extensive studies of low energy negative ion collision-induced dissociation of isotopically labeled hydroxylated-oxo-unsaturated fatty acids (34). However, determination of the structure based solely on the mass spectrum would not be possible. An unequivocal structure determination of the modification presumably requires labeling of the modification either with \(^{13}\)C for further NMR studies or with a radioactive isotope for easier detection and purification of the free modification. However, the practical problems with this approach should not be underestimated.

**Fig. 4.** Part of a two-dimensional \(^1\)H TOCSY NMR spectrum of the N-terminal tryptic peptide of post-translationally modified LTP1. The numbers on the two axes indicate chemical shifts in ppm. The cross-peaks shown here all originate from couplings between protons in the modification. Below, a plausible assignment of this spin system together with chemical shifts in ppm for individual protons. An ester bond between the modification and the LTP1-derived peptide is indicated.

**Fig. 5.** Assignments of spin systems originating from protons in the modification. The top four structures show assignments of observed spin systems. The numbers shown indicate chemical shifts in ppm. Below, our proposal for a structure compliant with the NMR data as well as the observed mass difference between ptm-LTP1 and um-LTP1 (294.3 Da).

**Fig. 6.** NMR evidence for a carboxylic acid in the LTP1 modification. A, DQF-COSY NMR spectrum (top) as well as a superposition of HMBC (circled cross-peaks) and HSQC NMR spectra. Chemical shifts in ppm are indicated on the two axes. The DQF-COSY spectrum shows proton-proton correlations from H2 to H3 and H4. The HSQC spectrum shows one-bond proton-carbon correlations, whereas the HMBC spectrum shows long range proton-carbon correlations. It should be noted that the HSQC cross-peak at δp 1.24 ppm and δc 24.2 ppm originates from another spin system. B, chemical structure highlighting the connectivities shown in panel A. Proton-proton (DQF-COSY) correlations are indicated by dotted lines. Proton-carbon correlations are indicated by full lines (HMBC) or broken lines (HSQC).
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Fig. 7. Electrospray ionization spectra in positive (A) and negative (B) ion mode of a sample prepared by acid-catalyzed hydrolysis of the bond between the N-terminal tryptic peptide and the modification. In positive ion mode, signals are observed for MH$_2$$^+$ and MHNa$_2$$^+$ of the unmodified peptide as well as a signal from a sodium adduct of the free modifying group (M*Na$^+$). In negative ion mode, signals for (M-H)$_2$$^+$ for the unmodified peptide as well as for (M-H)$^+$ for the free modifying group are observed. Selection and collision-induced dissociation of the latter ion results in the spectrum shown in panel C.

DISCUSSION

The function of plant ns-LTPs has for a long time been puzzling. The original suggestion that these proteins would act as intracellular lipid transporters was inconsistent with the observation that ns-LTPs are extracellularly located secretory proteins. Since then, most suggestions for their involvement in various aspects of plant physiology have been supported only by indirect evidence. Biochemically it has been found that ns-LTPs are able to bind lipids and catalyze their transfer by indirect evidence. Biochemically it has been found that ns-LTPs are able to bind lipids and catalyze their transfer in vitro, and it has been suggested that this may be an important part of the in vivo function. However, because ns-LTPs bind a large variety of lipids, it has been unclear which ligands, if any, are relevant for a physiological function. The work presented here is the first demonstration that an ns-LTP is bound to a lipid-like compound in planta.

We originally purified the LTP1 protein for other purposes. Initial mass spectrometric experiments revealed the presence of two peaks, one corresponding to um-LTP1 and a much larger peak corresponding to a hitherto undescribed modified form, ptm-LTP1. Because there presently is no good method of quantifying the relative amounts of the two forms in a crude extract (several types of gel electrophoresis have been tested), we have not searched for such enzyme activities.

We see no way that ptm-LTP1 could be an experimental artifact. Other authors have noticed the presence of a modified form of LTP1 with a mass just below 10.0 kDa (35–37). Additionally, a wheat homologue of barley LTP1 has been shown to exist in a post-translationally modified form having an excess mass of 294 Da (38). However, in none of these studies has the modifying entity been characterized in any detail nor has the modified amino acid been identified.

Post-translational modifications can be involved in many aspects of protein function, ranging from stability and dynamics to localization, regulation, and enzymatic activity. Also modifications of proteins by lipids are well known (39). However, none of these standard modifications can explain our observations. Furthermore, we have not been able to find any other instances of post-translational modifications in which lipid-like structures are bound through an oxygen ester bond to an acidic side chain of a protein. Thus, here we do not only describe the discovery of a modified form of LTP1 but also the finding of a new type of post-translational modification.

First we attempted to isolate the modification by cleaving the linkage to the protein and extracting the modification into an organic phase (not shown). Analysis of such an extract by MS yielded a peak, which may have originated from the modification. However, because ns-LTPs bind a large variety of lipids, it has been unclear which ligands, if any, are relevant for a physiological function. The work presented here is the first demonstration that an ns-LTP is bound to a lipid-like compound in planta.

We originally purified the LTP1 protein for other purposes. Initial mass spectrometric experiments revealed the presence of two peaks, one corresponding to um-LTP1 and a much larger peak corresponding to a hitherto undescribed modified form, ptm-LTP1. Presently we have no good explanation as to why this modification has escaped attention in earlier work. The samples used for the previous NMR structure determinations of LTP1 have been examined and found devoid of ptm-LTP1. However, other in-house samples contain ptm-LTP1. One explanation for this variance could be that earlier purification procedures were in a much larger scale. Therefore, several of the initial steps in the purification procedure would have taken much longer. It is possible that esterases, which could catalyze the hydrolysis of the protein-modification bond, are present in the barley flour extract. This could give large variations in the yield of um-LTP1 versus ptm-LTP1. Because there presently is no good method of quantifying the relative amounts of the two forms in a crude extract (several types of gel electrophoresis have been tested), we have not searched for such enzyme activities.

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First we attempted to isolate the modification by cleaving the linkage to the protein and extracting the modification into an organic phase (not shown). Analysis of such an extract by MS yielded a peak, which may have originated from the modification. However, both NMR and mass spectra of this sample show that it is heterogeneous, presumably because of side reactions during the cleavage and/or isolation procedure. However, by tryptic digestion a modified peptide, which can be detected by UV absorbance, was produced under mild conditions. By tandem MS sequencing of this peptide, we were able to localize aspartic acid 7 in the LTP1 sequence as the site of modification. Fig. 8 shows the structure of LTP1. As seen, Asp-7 is solvent-exposed with the carboxylate pointing toward the solvent. However, it should be remembered that the structure has been determined using um-LTP1. NOESY spectra of ptm-LTP1 indicate that the modification is localized inside the hydrophobic cavity (data not shown). This is not in contradiction to the structure in Fig. 8 because it can easily be envisaged that the energy gain of transferring the modification to the cavity may overcome the energy cost of turning helix 1 to make Asp-7 point more inward. In fact, it is reasonable that Asp-7 is solvent-exposed in um-LTP1 because it then becomes accessible for whatever machinery that may be necessary for attaching the modification to the protein.

Fig. 8. Structure of barley LTP1 showing the position of aspartic acid 7. The figure was prepared from PDB entry 1LIP (10) using MOLSCRIPT (45). The side chain of Asp-7 is shown as ball-and-stick. It should be remembered that the structure has been solved using unmodified LTP1.
By means of two-dimensional NMR, it was possible to obtain much information about the chemical nature of the modification. The structure shown in Fig. 5 fits all our experimental data including the observed mass difference between ptm-LTP1 and um-LTP1 (294.3 ± 0.1 Da). It is surprising that this compound contains an odd number of carbons (C_{17}H_{28}O_{5}). Although C_{17} lipids are known to occur naturally, they are much less common than even-numbered carbon chains. However, we have not been able to find any C_{2n} structures that can explain all our observations. The suggested structure of the modification is a substituted form of cis-7-heptadecenoic acid. This is highly interesting because cis-9-heptadecenoic acid (CHDA) and substituted forms thereof are known to possess antimicrobial activity toward fungi (40, 41) and bacteria (42). Plant ns-LTPs have been classified as pathogenesis-related proteins (43), and ns-LTPs from several species including barley (4) are known to inhibit the growth of pathogens in vitro. It is therefore relevant to note that CHDA and derivatives thereof have been isolated as the active compounds from the biocontrol agent Pseudozyma flocculosa (syn.: Sporothrix flocculosa), a yeast-like fungus with antagonistic activity against powdery mildew (40, 41). The mode of action of the biocontrol agent seems to be through the secretion of CHDA, which then interferes with the membranes in the attacked fungi, thereby causing rapid collapse of conidial chains (44). Although speculative, it is possible that the biological role of ns-LTPs may include antibiotic activity mediated through either protein-bound lipids or release of lipids as response to pathogens.

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