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Identification of Bitter Off-Taste Compounds in the Stored Cold Pressed Linseed Oil

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Whereas freshly pressed linseed oil provides a delicate nutty flavor, a lingering bitter off-taste is developing upon storage at room temperature. Using a sensory guided fractionation approach, the key bitter compound was identified in stored linseed oil, and its structure was determined as the methionine sulfoxide-containing, cyclic octapeptide *cyclo*(PLFIM *O*LVF) by means of FTIR, LC-MS, NMR spectroscopy, and amino acid analysis. Although this peptide is known in the literature as cyclolinopeptide E, the bitter taste activity of this compound has not previously been described. Sensory evaluation revealed a recognition threshold concentration of 12.3 μmol/L water.

KEYWORDS: Bitter taste; cyclolinopeptide; linseed oil; off-flavor; taste dilution analysis

INTRODUCTION

Over centuries linseed oil obtained from seeds of *Linum usitatissimum* L. is used as a dietary source of essential fatty acids in human nutrition. Today's production of linseed oil reaches about 2.5 million metric tons with Canada, China, and the United States of America ranking in the first places (*1*). In Germany, the production of linseeds decreased sharply in 2001 due to changed agricultural subsidies and has been cultivated during the past years on about 10000–16000 ha in 2006 (*2*). Linseed oil offers a high amount of polyunsaturated fatty acids, with 57% α-linolenic acid and 15% linoleic acid in the total fatty acids (*3*). When stored under appropriate conditions, the oil is rather stable in the intact seed for more than 1 year. But after preparing the linseed oil by pressing, the shelf life of the oil obtained is limited to only about 3–6 months mainly depending on storage and packaging conditions. While the freshly pressed linseed oil provides a delicate nutty, mild roasted flavor, an unpleasant bitter off-taste starts to develop already after 1 day of storage.

However, the development of this bitter off-taste could be prevented upon refining even after a storing period of several months (preliminary experiments, data not shown). Also, endogeneous cyanogenic glycosides in the seeds such as linamarin, linustatin, neolinustatin, and lotaustralin have been suggested as key bitterness inducers (*4*). Furthermore, lignanes might contribute to the bitterness of linseed oil. Lignanes such as matairesinol, lariciresinol, and isolariciresinol have been identified in linseeds (*5*). They are found in the hull of the seeds and have to be separated from the freshly pressed oil by sedimentation or filtration as the solids or "foots" (*6*). But model experiments performed with linseed oil stored with or without contact to the press cake revealed no significant difference in the bitter taste impact perceived. As reported for olive oil, also the role of phenolic compounds like *p*-cumaric acid, caffeic acid, sinapic acid, and ferulic acid in bitterness of linseed oil cannot be excluded (*7, 8*). Alternatively to these phenolic secondary plant metabolites, bitter tasting hydroxylated fatty acids such as 9,12,13-trihydroxyoctadec-10- and 9,10,13-trihydroxyoctadec-11-enoic acids, which have been identified in stored soy bean oil (*9*), might account for the off-taste developing during storage of linseed oil upon lipid peroxidation. Taking all the literature information into consideration, it has to be concluded that the key drivers for the bitter taste of stored linseed oil are yet not clear.

Therefore, the objective of the present study was to (i) evaluate the bitter taste developing upon storage of freshly pressed linseed oil varying storage temperature and packaging conditions and (ii) to identify the most intense bitter compound responsible for the bitter taste in stored cold pressed linseed oil.

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MATERIALS AND METHODS

Chemicals and Materials. Linseed oils were purchased from a local supplier or were obtained from the Dörnthaler oil mill (Pfaffroda-Dörnthal, Germany) and Alberdingk Boley GmbH (Düsseldorf, Germany, refined linseed oils). In addition, linseed oil was freshly prepared from Canadian seeds by means of a laboratory screw press (IBG Monforts, Mönchengladbach, Germany). The seeds were pressed with an 8 mm nozzle at a temperature not exceeding 60 °C, and the speed of rotation of the screw was 35 rpm. The temperature of the freshly obtained oil (30% yield) did not exceed 40 °C. All solvents were of HPLC grade (Merck, Germany). The water used was either distilled twice or was HPLC grade. Deuterated solvents were obtained from Euriso-top (Gif-Sur-Yvette, France).

Storage Experiments. Immediately after preparation, the fresh crude linseed oil was filtered by pressing through a 2 μm pore size membrane filter and stored in aliquots (50 mL) in brown glass bottles avoiding any headspace at −18 and 20 °C.

Sensory Evaluation of the Bitter Taste of Linseed oil. The bitterness of the oil samples was evaluated by five trained sensory panelists using serial dilutions of the oil in odor and tasteless refined rapeseed oil (undiluted, 1 + 1, 1 + 2, 1 + 3, 1 + 4, and so on). The panelists have given the informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders. A portion (15 g) of each dilution was presented in order of ascending concentrations of the linseed oil amount in coded blue tasting glasses to each taster at room temperature. Using the sip-and-spit method, the panelists proceeded with tasting the next higher concentration until the bitter recognition threshold was reached. This dilution was defined as taste dilution (TD) factor (*10*). The TD factors evaluated by sensory panel in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step.

Extraction of the Bitter Compound from Linseed Oil. An aliquot (100 g) of the bitter linseed oil was dissolved in heptane (100 mL) and then extracted three times with methanol/water (6/4; v/v; 200 mL each). The combined aqueous layers were filtered through a wet filter paper to separate traces of oil from the layer, and the solvent was removed in vacuum. Sensory analysis of an aqueous solution of the residue obtained demonstrated that an intensely bitter tasting fraction has been isolated form linseed oil.

Silica Gel Fractionation of the Bitter Extract. The bitter fraction obtained from the oil was dissolved in methanol/diethyl ether (1/1, v/v; 1 mL) and placed onto the top of a glass column (2 × 15 cm, 50 mL reservoir) filled with a slurry of silica gel (20 g) deactivated with water (5%, w/w) and conditioned with diethyl ether. Chromatography was performed with solvent mixtures (20 mL) using diethyl ether and ethanol in ratios (v/v) of 10/0 (fraction 1), 9/1 (fraction 2), 4/1 (fraction 3), 7/3 (fraction 4), 3/2 (fraction 5), 1/1 (fraction 6), 2/3 (fraction 7), 3/7 (fraction 8), 1/4 (fraction 9), 1/9 (fraction 10), and 0/10 (fraction 10). The individual fractions obtained were freed from solvent under vacuum; the residues were taken up in water (3 mL) and then evaluated for bitter taste by means of the trained sensory panel. The most intense bitter taste was detected in fractions 4–8.

Purification of the Key Bitter Principle in Fractions 4–8 by RP-18 HPLC. An aliquot (4.4 mg) of the material isolated from silica fractions 4–8 was dissolved in water/ethanol (1/1, v/v; 1.5 mL), and aliquots (100 μL) were separated by HPLC on a 250 × 4 mm, 5 μm, LiChrospher 100 RP-18 column (Merck, Germany) connected to a UVD 340-type UV/vis detector (Gynkotek, Germany) operating at a wavelength of 210 nm. Chromatography was performed starting with a mixture of methanol/water (75/25, v/v) and then increasing the methanol to 100% within 25 min. The effluent was collected in 1 min fractions by means of a L-5200 fraction collector (Merck-Hitachi, Germany), each fraction was freed from solvent, the residues obtained were taken up in water (3 mL) upon ultrasonification, and the individual solutions were evaluated for bitter taste by means of the sensory panel.

In order to obtain enough material for structure elucidation and amino acid analysis, higher amounts of the intensely bitter tasting material present in LC fractions 4–8 were isolated and purified by using a 250 × 20 mm i.d., LiChrospher 100 RP-18 column (Knaur, Germany) connected to a preparative HPLC system consisting of two K-1800 pumps (Knaur, Germany), a K-2501 UV/vis detector (Knaur, Germany), and a model 201 fraction collector (Gilson, UK). Using a flow rate of 25 mL/min and monitoring the effluent at 210 nm, the intensely bitter tasting principle was isolated as a white amorphous powder and was identified as *cyclo*(Pro-Leu-Phe-Ile-Met *O*-Leu-Val-Phe), known as cyclolinopeptide E (*11*), by means of LC-MS, ESI-MS-TOF, and NMR analysis.

Cyclolinopeptide E, *cyclo*(Pro-Leu-Phe-Ile-Met *O*-Leu-Val-Phe), *cyclo*(PLFIM *O*LVF): LC-MS: molecular weight 976.7 represented by *m/z* 977.7 $[M + H]^+$, 994.7 $[M + NH_4]^+$, 999.7 $[M + Na]^+$, 1015.7 $[M + K]^+$; 489.5 $[M + H]^{2+}$, 500.5 $[M + H + Na]^{2+}$, 508.4 $[M + H + K]^{2+}$, 511.5 $[M + 2Na]^{2+}$. ESI-MS-TOF (positive): $[C_{51}H_{76}N_8O_9S + Na]^+$: measured: *m/z* 999.5366, calculated: *m/z* 999.5348; ESI-MS-TOF (negative): $[C_{51}H_{76}N_8O_9S - H]^+$: measured: *m/z* 975.5390, calculated: *m/z*975.5383. ^{1}H NMR (500 MHz, DMSO-d_6, COSY, NOESY): Pro^1: δ 4.37 (1H, m, α), 1.83 (1H, m, β), 2.17 (1H, m, β), 1.91 (2H, m, γ), 3.50 (1H, m, δ), 3.56 (1H, m, δ); Leu^2: δ 3.86 (1H, m, α), 1.57 (1H, m, β), 1.77 (1H, m, β), 1.66 (1H, m, γ), 0.79 (3H, m, δ_{Me}), 0.91 (3H, m, δ_{Me}), 8.18 (1H, br s, NH); Phe^3: δ 4.42 (1H, m, α), 2.81 (1H, m, β), 3.34 (1H, m, β), 7.11 (2H, m, ε), 7.15 (1H, m, ζ), 7.23 (m, 2H, δ), 7.80 (1H, br s, NH); Ile^4: δ 3.87 (1H, m, α), 1.38 (1H, m, β), 1.48 (1H, m, γ), 1.02 (1H, m, γ), 0.86 (3H, m, δ_{Me}), 0.98 (3H, d, 6.8 Hz, γ_{Me}), 7.90 (1H, br s, NH); Met *O*^{5}: δ 4.22 (1H, m, α), 2.01 (1H, m, β), 2.19 (1H, m, β), 2.67 (1H, m, γ), 2.97 (1H, m, γ), 2.54 (3H, s, ε_{Me}), 8.04 (1H, br s, NH): Leu^6: δ 4.51 (1H, m, α), 1.95 (1H, m, β), 2.51 (1H, m, β), 1.97 (1H, m, γ), 0.79 (3H, m, δ_{Me}), 0.89 (3H, m, δ_{Me}), 7.75 (1H, br s, NH); Val^7: δ 3.77 (1H, m, α), 1.98 (1H, m, β), 0.63 (3H, d, 6.6 Hz, γ_{Me}), 0.72 (3H, d, 6.6 Hz, γ_{Me}), 7.75 (1H, br s, NH); Phe^8: δ 4.93 (1H, m, α), 2.92 (1H, m, β), 3.12 (1H, m, β), 7.15 (1H, m, ζ), 7.20 (2H, m, δ), 7.24 (2H, m, ε), 8.53 (1H, br s, NH). ^{13}C NMR (125 MHz, DMSO-d_6, HMQC, HMBC): Pro^1: δ 62.1 (α), 28.9 (β), 24.2 (γ), 47.3 (δ), 170.3 (C═O); Leu^2: δ 54.8 (α), 38.8 (β), 24.1 (γ), 22.5 (δ_{Me}), 21.1 (δ_{Me}), 172.1 (C═O)); Phe^3: δ 52.8 (α), 35.4 (β), 137.1 (γ), 127.8 (δ), 128.5 (ε), 126.0 (ξ), 170.6 (C═O)); Ile^4: δ 53.4 (α), 38.4 (β), 24.5 (γ), 16.6 (γ_{Me}), 10.1 (δ_{Me}), 172.4 (C═O)); Met *O*^{5}: δ57.2 (α), 24.15 (β), 49.7 (γ), 38.0 (ε_{Me}), 170.0 (C═O)); Leu^6: δ 51.4 (α), 24.7 (β), 28.8 (γ), 22.5 (δ_{Me}), 21.4 (δ_{Me}), 171.8 (C═O)); Val^7: δ 59.4 (α), 29.9 (β), 19.2 (γ_{Me}), 18.4 (γ_{Me}), 171.3 (C═O)); Phe^8: δ 51.2 (α), 36.4 (β), 138.1 (γ), 127.9 (δ), 129.1 (ε), 126.0 (ξ), 170.9 (C═O)).

Amino Acid Analysis. An aliquot (300 μg) of the isolated bitter principle was dissolved in aqueous hydrochloric acid (6 mol/L; 100 μL) and heated for 17 h at 110 °C under an atmosphere of nitrogen. An aliquot (10 μL) of the hydrolysate was injected into a Dionex BioLC system equipped with a 250 × 2 mm Dionex AminoPac PA10 column with precolumn, a GS50-type gradient pump, an AS50-type autosampler, an AS50-type thermal compartment with column switching valve, and a pulsed amperometric ED50 gold electrode as well as a pH//Ag/AgCl reference electrode. Chromatography (0.25 mL/min) was performed by using the following gradient including deionized water (solvent A), aqueous sodium hydroxide (250 mM/L, solvent B), aqueous sodium acetate (1 mol/L; solvent C), and aqueous acetic acid (100 mmol/L; solvent D): solvent mixture A/B (76/24, v/v) for 2 min, then raised to A/B (64/36, v/v) for 6 min, maintained for 3 min, then changed to solvent mixture A/B/C (40/20/40, v/v/v) for 3 min, then raised to A/B/C (44/16/40, v/v/v), maintained for 3 min, raised to A/B/C (14/16/70, v/v/v) maintained for 22 min, and, finally, changed to 100% of solvent D. Identification of the individual amino acids was performed by comparison of the retention time with those of the reference compounds as well as by cochromatography.

Fourier Transformation Infrared Spectroscopy (FTIR). A mixture of dry potassium bromide (250 mg) and an aliquot (1.5 mg) of the isolated bitter principle was pressed under vacuum to give a clear pellet of 13 mm i.d. The IR spectrum was recorded on an IFS-28-type FTIR spectrometer (Bruker, Germany) from 4000 to 400 cm^{-1}.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, COSY, NOESY, ^{13}C, HMQC, and HMBC experiments were performed on an Avance-500 (Bruker, Rheinstetten Germany). Using methanol-d_4 or DMSO-d_6 as solvents, chemical shifts were measured from using tetramethylsilane (TMS) as the internal standard.

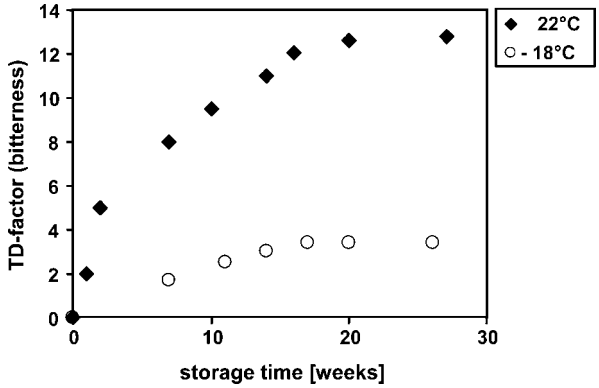

Figure 1. Influence of storage on the development of a bitter off-taste in freshly prepared linseed oil.

Liquid Mass Spectrometry (LC-MS). A portion (100 μg) of the isolated bitter principle was solved in methanol (1 mL), and an aliquot (5 μL) was injected into an API 4000 Q Trap LC/MS/MS (Applied Biosystems Sciex Instruments, Darmstadt, Germany) by means of loop injection with methanol/water (1/1, v/v) as the solvent (200 μL/min). The MS was operated in the electrospray ionization (ESI) mode at 350 °C ionization temperature using positive polarity 5500 V ion spray voltage. System parameters for curtain gas (20 psi), nebulizer gas (30 psi), heater gas (45 psi), fragmentation gas nitrogen ($p = 4.2 \times 10^5$ Torr), declustering potential (+50 V), and collision energy (+30 V) are given in parentheses.

High Resolution Mass Spectrometry. A portion (100 μg) of the bitter isolate was solved in methanol (1 mL) and an aliquot (5 μL) was injected into a Bruker Mikro TOF (Bruker, Daltonik, Bremen, Germany) using electrospray ionization in positive and negative mode by means of loop injection with methanol/water (1/1, v/v) as the solvent (200 μL/min). The mass scale was recalibrated with sodium formate cluster peaks from a reference injection performed prior to the sample measurement. The results differed less than 5 mDa in a range from 100 to 3000 *m*/*z*.

RESULTS AND DISCUSSION

In order to identify the compounds responsible for the development of bitter-off taste during storage of linseed oil, linseed was pressed by means of a laboratory screw press to obtain a fresh crude oil with a yielded of about 30%. After filtration, the oil exhibited a yellowish-golden color and provided a delicate nutty and toasted bread-like flavor without any bitter off-taste.

Influence of Storage on Bitter Taste Development. To visualize the influence of storage on bitter taste development, the freshly prepared linseed oil was stored in the dark in sealed bottles at −18 and 22 °C for up to 30 weeks. Individual samples, taken after various storage periods, were rated in their bitter impact by using a taste dilution analysis (*10*). To achieve this, serial dilutions of the linseed oil in odor and tasteless refined rapeseed oil were presented in order of ascending concentrations of the linseed amount to a trained sensory panel which was asked to determine the so-called taste dilution (TD) factor at which the bitter taste could just be detected. As given in **Figure 1**, sensory analysis revealed that in the oil sample stored at 22 °C a lingering bitter taste developed after an induction period of about 2 days and approximated a maximum TD factor of 13 after about 20 weeks. In comparison, the linseed oils stored at −18 °C reached a significant lower bitter taste impact; e.g., after 20 weeks of storage a TD factor of 4 was determined. These data clearly demonstrate that the lingering bitter taste is developing during storage of freshly pressed linseed oil at room temperature whereas storage at −18 °C reduces significantly the formation of bitter compounds. This might open a new way to sell linseed oil as a frozen food.

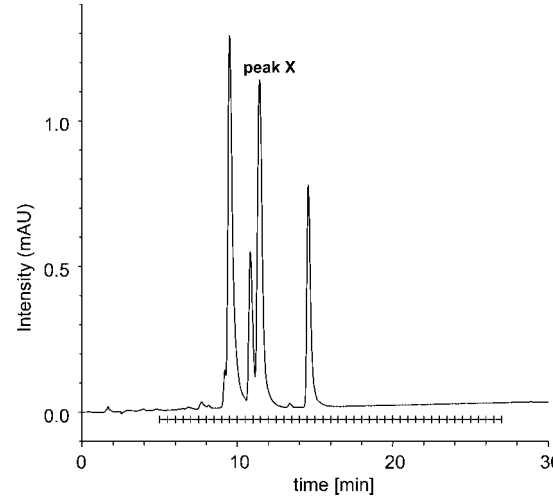

Figure 2. RP-HPLC chromatogram of a bitter-tasting isolate obtained from stored linseed oil.

Isolation of the Key Bitter Principle. A sample of the bitter linseed oil was diluted with heptane and then extracted with a methanol/water mixture. The aqueous layer was freed from solvent to yield about 55 mg of a pale yellow amorphous powder from 100 g of oil. Sensory evaluation of this material dissolved in water revealed an intense and lingering bitter taste of the isolate. In contrast, the extracted linseed oil which was freed from solvent in high vacuum at 80 °C did not show any bitter taste (data not shown), thus demonstrating that all the bitter compounds have been successfully isolated by the methanol/water extraction.

The bitter-tasting extract was further fractionated by means of column chromatography on silica gel into 10 subfractions using diethyl ether/ethanol mixtures of increasing polarity. After removing the solvents under vacuum, sensory experiments revealed that fractions 4–8 exhibited the most intense bitterness. These bitter fractions were collected and further separated on a preparative reversed phase HPLC column as outlined in **Figure 2**. Monitoring the effluent at 210 nm, 44 HPLC fractions were automatically collected, freed from solvent, and again evaluated sensorially in water. HPLC fractions 13/14 containing the effluent of the peak X detected at 11.4 min exhibited by far the highest bitter taste intensity whereas all the other fractions were either less bitter or entirely tasteless. From peak X, about 11 mg of a bitter principle was isolated from 100 g of linseed oil. The UV/vis spectrum of that bitter compound eluting after 11.4 min showed an absorption maximum at 210 nm with a small shoulder at 260 nm.

Structure Elucidation of the Key Bitter Principle. In order to identify the chemical structure of the isolated bitter compound, the isolate was analyzed by means of FTIR (**Figure 3**). A broad band at 3427.7 cm^{-1} corresponds to a hydroxyl group with a shoulder at 3314.0 cm^{-1} for a NH vibration. The presence of a hydroxylic group is in good agreement with the good extractability of the bitter compound from the oil. The bands of CH_3 and CH_2 groups are detectable at 2959.3 and 2928.3 cm^{-1}. Interestingly, the intensity of both signals is very low and the ratio is about 1:1, thus indicating a lack of long carbon chains and excluding some fatty acid derivatives as bitter compound. Furthermore, bands at 1659.1 and 1529.9 cm^{-1} correspond to C═O (vibration) and C–N, N–H (vibration) for *N*-monosubstituted amides, while bands at 1030.6 cm^{-1} and at 746.0 and 701.3 cm^{-1} correspond to sulfoxide (vibration) and a monosubstituted aromatic system, respectively. Usually, monosubstituted aromatic compounds provide two signals of equal

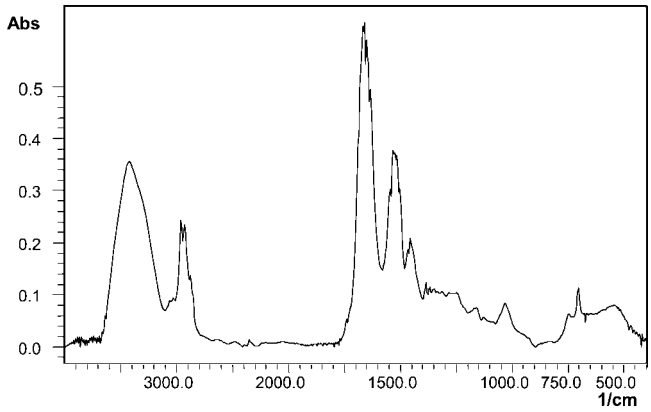

Figure 3. FTIR spectrum of the isolated key bitter compound (peak X in **Figure 2**) in KBr pellet.

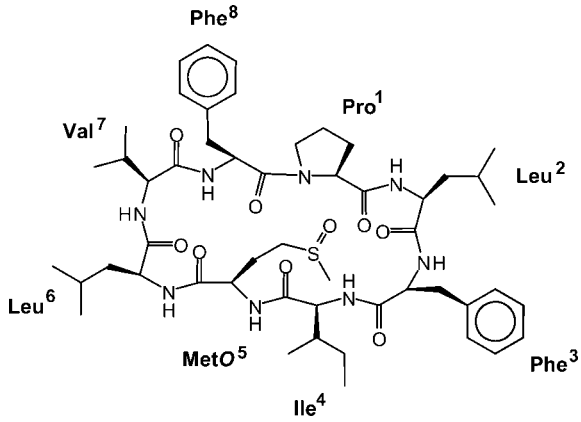

Figure 4. Structure of cyclolinopeptide E consisting of L-proline (Pro), L-leucine (Leu), L-phenylalanine (Phe), L-isoleucine (Ile), L-methionine sulfoxide (MetSO), and valin (Val).

intensity, but for the present compound the band at 746.0 cm^{-1} is rather less intense (*12*). The indication for the aromatic system is in agreement with the UV spectrum from the diode array detector of the HPLC system.

For more detailed structure elucidation, the molecular mass of the bitter compound has been determined by means of LC-MS running in the positive ionization mode. Besides the pseudomolecular ion with *m/z* 977.7 $[M + H]^+$, product cluster ions were observed for ammonium with *m/z* 994.7 $[M + NH_4]^+$, sodium with *m/z* 999.7 $[M + Na]^+$, and potassium with 1015.7 $[M + K]^+$. In addition, a similar pattern was observed for the double charged molecule with 489.5 $[M + 2H]^{2+}$, 500.5 $[M + H + Na]^{2+}$, 508.4 $[M + H + K]^{2+}$, and 511.5 $[M + 2Na]^{2+}$. In a next step exact mass measurements were done for the sodium cluster ion $[M + Na]^+$ (*m/z* 999.5366) in the positive

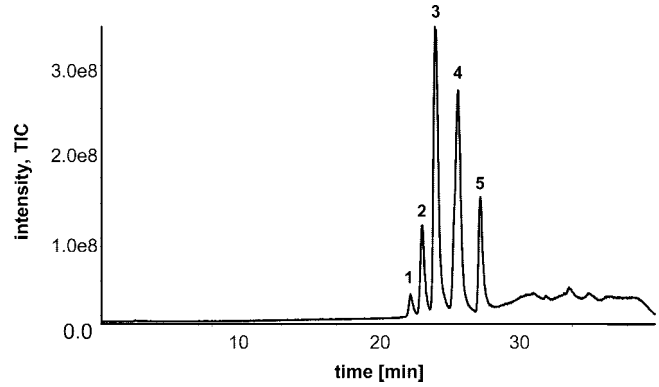

Figure 5. LC-MS (TIC) chromatogram of a bitter isolate obtained from stored linseed oil.

ionization mode showing best fit for the ion $[C_{51}H_{76}N_8O_9S + Na]^+$ (calcd *m/z*999.5348). Measurements in the negative ionization mode revealed a pseudomolecular ion $[C_{51}H_{76}N_8O_9S - H]^-$ (*m/z* 975.5390) deviating just 0.7 mDa from the calculated *m/z* 975.5383, thus demonstrating $C_{51}H_{76}N_8O_9S$ as the elementary composition of the isolated bitter compound. The eight nitrogen atoms in the molecular formula gave some evidence for a peptide structure. In addition, the hydrophobicity as well as surprisingly lacking amino acid fragmentation from the terminal of the peptide chain upon LC-MS analysis might indicate the existence of a cyclic peptide.

In order to support the hypothesis of a bitter peptide, an aliquot of the sample was hydrolyzed and then analyzed by means of ion chromatography for its tentative amino acid composition. Six amino acids were identified, namely, L-proline, L-valine, L-leucine, L-isoleucine, L-phenylalanine, and L-methionine sulfoxide. This amino acid composition matched perfectly with the structure of the cyclic octapeptide Pro-Leu-Phe-Ile-MetO-Leu-Val-Phe, given in **Figure 4**, which has been described as cyclolinopeptide E (CLE) in the literature to be present in linseed (*11*).

For a final confirmation of the proposed structure, ^1H NMR data were recorded for the bitter compound. The ^1H NMR spectrum revealed a total of eight α-amino acid protons resonancing between δH 3.7 and 5.0, fitting well with the proposed structure of a cyclic octapeptide. In addition, seven amide protons could be observed, indicating the presence of one proline in the molecule. The signal at δH 2.54 with an intensity of three protons is fitting well with the methyl group of the methionine sulfoxide. In the δH range between 7.1 and 7.3 the expected aromatic signal pattern of the two phenylalanine moieties was observed. Moreover, ^{13}C NMR spectroscopy showed eight carbonyl signals and, in addition, the two typical

Table 1. Cyclolinopeptides Present in Linseed Oil

peak no.[a]	cyclolinopeptide (CL)[b]	measured masses	literature data[c]	pseudomolecularions
1	CLF	1084.8	1084.5	$[M + H]^+$
	cyclo(PFFTVMOLMO)	1106.7	1106.7	$[M + Na]^+$
2	CLG	1098.7	1098.5	$[M + H]^+$
	cyclo(PFFTIMOLMO)	1120.7	1120.5	$[M + Na]^+$
3	CLC	1074.7	1074.6	$[M + H]^+$
	cyclo(PPFVIMOLI)	1096.7	1096.6	$[M + Na]^+$
4	CLE	977.8	977.5	$[M + H]^+$
	cyclo(PLFIMOLVF)	999.8	999.5	$[M + Na]^+$
5	CLA	1040.9	1040.6	$[M + H]^+$
	cyclo(PPFFLIILV)	1062.8	1062.6	$[M + Na]^+$

[a] Peak numbering refers to Figure 5. [b] Abbreviation of amino acids: L-proline (P), L-phenylalanine (F), L-tryptophane (T), L-valine (V), L-methionine sulfoxide (MO), L-leucine (L), and L-isoleucine (I). [c] Literature data are taken from refs *8*, *9*, and *16*.

δC shifts at 137.1 and 138.1 expected for the quarternary carbons of the aromatic ring in the phenylalanine residue. HMBC correlations between the amide carbonyl atoms and the neighboring amide protons as well as the α-amino acid protons and, in addition, NOE correlations between neighboring amino acids gave a complete picture of the structure. Comparison of the NMR data obtained for the bitter compound with those reported earlier (*11*) led to the unequivocal identification of the bitter tastant as cyclinopeptide E. The human recognition threshold for bitterness was determined in bottled water using a triangle test to be 12.3 μmol/L in water. Although this peptide has been analyzed for its biological activity such as its immunosuppressive activity (*13*), the intense bitter taste of this peptide has not been reported to date.

Additional Cyclopeptides in Linseed Oil. Besides *cyclo*-(PLFIM *O*LVF), cyclolinopeptide E, there are literature reports on the existence of other cyclolinopeptides named cyclolinopeptide A to I with some differences in the amino acid number (octa- or nonapeptides), composition, or sequence (*6*, *11*, *14*–*19*) In order to gain some preliminary insights into the potential role of these peptides in linseed bitterness, the bitter tasting aqueous methanolic extract isolated from linseed oil was analyzed for their pseudomolecular ions by means of LC-MS. As outlined in **Figure 5**, five potential cyclopeptide candidates were detected. Compound **4** was again identified as the intense bitter tasting CLE. The MS data show evidence that peaks 1, 2, 3, and 5 are the cyclolinopeptides CLF, CLG, CLC, and CLA, respectively (**Table 1**). Their compositions have been analyzed to be *cyclo*(PPFFLIILV) named cyclolinopeptide A, *cyclo*-(PPFVIM *O*LI) named cyclolinopeptide C, *cyclo*(PFFTVM *O*LM *O*) named cyclolinopeptide F, and *cyclo*(PFFTIM *O* LM *O*) named cyclolinopeptide G (*11*, *14*). Interestingly, these peptides seem not to be of key importance for linseed oil bitterness as demonstrated by HPLC-tasting experiments (**Figure 2**).

In conclusion, the bitter compound formed in cold pressed linseed oil is reported for the first time to be cyclolinopeptid E at the threshold concentration of 12.3 μmol/L (water). Since linseed is also known to contain cyclopeptide *cyclo*(PLFIMLVF) (*19*, *20*), which differs just by the methionine moiety instead of the methionine sulfoxide, the formation of the bitter peptide might be due to an oxidation of the sulfur atom in the precursor peptide. Studies on clarifying the mechanism of that oxidation step as well as sensory studies on the activity of such cyclopeptides in various oil matrices are currently under investigation.

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