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Isolation and Identification of Trichothecenes from *Fusarium compactum* Suspected in the Aetiology of a Major Intoxication of Sandhill Cranes

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Isoneosolaniol (4,8-diacetoxy-12,13-epoxytrichothec-9-ene-3,15-diol) and other unidentified trichothecene mycotoxins were isolated from culture extracts of two highly toxigenic strains of *Fusarium compactum* cultured from waste peanuts involved in an acute intoxication of sandhill cranes (*Grus canadensis*). Neosolaniol and other unidentified trichothecenes were detected in waste peanuts collected from affected areas. The structure of isoneosolaniol was determined by ^{1}H and ^{13}C NMR analyses and by high-resolution mass spectrometry. Isoneosolaniol was highly toxic to 1-day-old chickens and to a HEp2 cell culture assay. It was concluded that the most logical cause of the sandhill crane intoxication was *Fusarium* spp. contaminated peanuts and various trichothecene mycotoxins acting alone or in conjunction with other *Fusarium* mycotoxins.

An estimated 9075 sandhill cranes (*Grus canadensis*) died at or near a major roost site at Cedar Lake in Gaines County, TX, from 1982 to 1987, with a major loss (5000) occurring during January and February 1985 (Windingstad et al., 1987). Concurrent studies have implicated waste peanuts contaminated with *Fusarium* species as the most probable cause (Windingstad et al., 1988; Nelson et al., 1988). Prominent clinical signs in sick cranes were the inability to hold their heads erect and difficulty in flying. Cranes that could fly did so with head, neck, and legs drooped perpendicularly to the body. Submandibular edema observed in sick and dead cranes may have resulted from this prolonged posture of the head.

Peanuts were the major component of the cranes' diet, with over 95% of the dead cranes having peanuts in their gizzards (Windingstad et al., 1988). Nelson et al. (1988) have shown that *Fusarium* was the major fungal genus on waste peanuts collected from the affected area. Although several of the species isolated (*Fusarium moniliforme*,

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Fusarium proliferstum, *Fusarium solani*, etc.) are known to be producers of mycotoxins, the most toxigenic species isolated was *Fusarium compactum* (Nelson et al., 1988).

This paper reports the isolation and identification of the major trichothecene mycotoxin produced by isolates of *F. compactum* and the presence of trichothecene mycotoxins in waste peanuts collected from the site of the intoxication.

EXPERIMENTAL SECTION

Isolation of Toxic Metabolites from *Fusarium* Cultures. Two highly toxigenic isolates of *F. compactum* isolated from waste peanuts involved in an acute intoxication of sandhill cranes (*G. canadensis*) were cultured on autoclaved peanuts (Nelson et al., 1988; Windingstad et al., 1987, 1988). Cultures were incubated at 25 °C for 3 weeks in the dark prior to extraction of toxic metabolites (Nelson et al., 1988). Both toxigenic fungal cultures were combined and extracted twice with an excess of chloroform–MeOH (2:1, v/v) with an Ultra Turrax homogenizer, filtered, and evaporated to dryness. The extract was partitioned between 80% aqueous methanol–*n*-hexane to remove oil. The aqueous methanol phase was evaporated under vacuum at 60 °C to remove the methanol and the aqueous portion partitioned against chloroform. The chloroform phase was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness.

The chloroform phase was redissolved in a minimal amount of benzene, applied to a silica gel 60 column (2.5 × 40 cm) packed in benzene, and eluted with a linear gradient from benzene to ethyl acetate (tubes 1–131) followed by elution with a linear gradient from ethyl acetate to acetone (tubes 132–233). Fractions of 20 mL were collected. On the basis of thin-layer chromatography (TLC) and bioassay, fractions 1–147, 148–176, and 177–233 were each combined. Fractions 148–176 contained the major toxic metabolite; these combined fractions were evaporated to dryness, applied to a C_{18} reversed-phase column (3.5 × 13 cm) packed in methanol, and equilibrated with 200 mL of water–methanol (80:20, v/v). The column was eluted with a linear gradient from water–methanol (80:20, v/v) to methanol. A total of 70 20-mL fractions were collected. The toxin was in tubes 32–37, which were combined and evaporated to dryness under vacuum at 60 °C.

The partially purified toxin was chromatographed on a Chromatotron (preparative centrifically accelerated, radial, thin-layer chromatograph) using a silica gel PF rotor (1-mm thickness) and eluted with benzene–ethyl acetate–acetic acid (5.5:4.0:0.5, v/v/v). A total of 33 3-mL fractions were collected. The purified toxin was found in tubes 19–33. These tubes were combined and evaporated to dryness to yield 102 mg of a thick oil.

Analysis of Waste Peanuts for Toxic Metabolites. A representative sample of waste peanuts (100 g) collected at the site of the intoxication was blended at high speed with chloroform (300 mL) for 3 min in an Ultra Turrax homogenizer. After filtration and centrifugation, the chloroform extract was evaporated to dryness to leave 30 g of crude material. A portion of this material (10 g) was dissolved in 60 mL of methanol–water (20:80) and extracted with hexane (3 × 10 mL), which was discarded. The methanol was removed from the aqueous phase on a rotary evaporator, and then the residue (ca. 50 mL) was adsorbed onto a Chemtube (Analytichem International, Harbor City, CA) and extracted with 300 mL of ethyl acetate. After removal of the ethyl acetate, the residue (4.4 mg) was analyzed directly by TLC, mass spectrometry, and, after dilution with acetone, HEp2 bioassay. Fractions purified by preparative TLC were also tested by bioassay.

Bioassays. Toxin purification was monitored on 1-day-old chickens according to the method of Kirksey and Cole (1974). The purified toxin, T-2 toxin, and neosolaniol (Makor Chemicals, Ltd., Jerusalem, Israel) were bioassayed by both plant and animal cell assays. The animal cell assay was carried out on a HEp_2 cell line grown on Hank's basal medium with Eagle's modification supplemented with 15% calf bovine serum. The relative degree of toxicity was established by assaying serial dilutions. The plant bioassay was conducted with coleoptile sections 4 mm long cut from 4-day-old wheat seedlings (*Triticum aestivum* L. cv. Wakeland) grown in the dark on moist trays of sand at 22 ± 1 °C (hancock et al., 1964). The wheat coleoptile bioassay measures effects of chemicals on plant growth promotion or inhibition.

Thin-Layer Chromatography. Thin-layer chromatography was performed on precoated silica gel 60 F-254 plates (5 × 10 cm; EM Laboratories, Inc., Elmsford, NY) developed with toluene–ethyl acetate–formic acid (5:4:1, v/v/v) or with chloroform–methanol (95:5). The toxin was visualized as a peach spot after spraying, first with 1% (*p*-dimethylamino)benzaldehyde in ethanol and then with 50% ethanolic sulfuric acid followed by heating for 2 min at 100 °C.

A general spray test used to indicate the presence of trichothecenes was 4-(*p*-nitrobenzyl)pyridine reagent sprayed on the TLC plates followed by heating in the presence of base (Takitani et al., 1979), when epoxy compounds are visualized as blue spots against a white background.

NMR Spectroscopy. The toxin was dissolved in deuteriochloroform, and proton NMR spectra were recorded at 200 MHz on a Nicolet NTC Fourier transform (FT) NMR spectrometer, chemical shifts being reported in parts per million relative to tetramethylsilane internal standard. Carbon-13 spectra were obtained at 50 MHz on a Nicolet NTC FT-NMR spectrometer.

Mass Spectrometry. Probe mass spectra were obtained on a VG 12000 quadrupole mass spectrometer by taking a portion of the extract diluted in acetone and heating in a glass tube of the insertion probe from 50 °C at 50 °C/min to 350 °C. Spectra were obtained in electron and chemical ionization scanning from 35 to 600 μ in 1 s.

GC/MS analysis was carried out with a Carlo Erba 4160 GC directly coupled to a VG 12000 quadrupole mass spectrometer. The 25 cm × 0.22 mm (i.d.) BP1 fused silica column (SGE, U.K.) was operated at a helium gas flow rate of 1 mL/min with split injection (1.5 μL, 20:1 split). The column temperature was programmed from 150 °C at 30 °C/min to 250 °C and then at 5 °C/min to 300 °C. The column outlet was connected directly to the mass spectrometer source held at 200 °C, and the mass spectrometer was operated in an electron ionization mode at 70 eV with a 200-μA trap current. Scanning was from $m/2$ 40 to 600 in 2 s.

Chemical ionization spectra were obtained with both ammonia and isobutane under identical conditions to those above at a source pressure of 5×10^{-5} Torr.

Thermospray spectra were obtained at a VG thermospray interface to a VG 12000 quadrupole mass spectrometer. The purified toxin fraction (1 mg) was dissolved in 300 μL of methanol–acetonitrile 0.1 M ammonium acetate (1:1:1) and directly injected (20 μL) without any chromatography into an isocratic solvent stream of 0.1 M ammonium acetate–acetonitrile. The thermospray interface was operated at a source temperature of 225 °C, a vaporizer temperature of 250 °C, and a chamber temperature of 260 °C. Spectra were obtained in a positive-ion mode, with

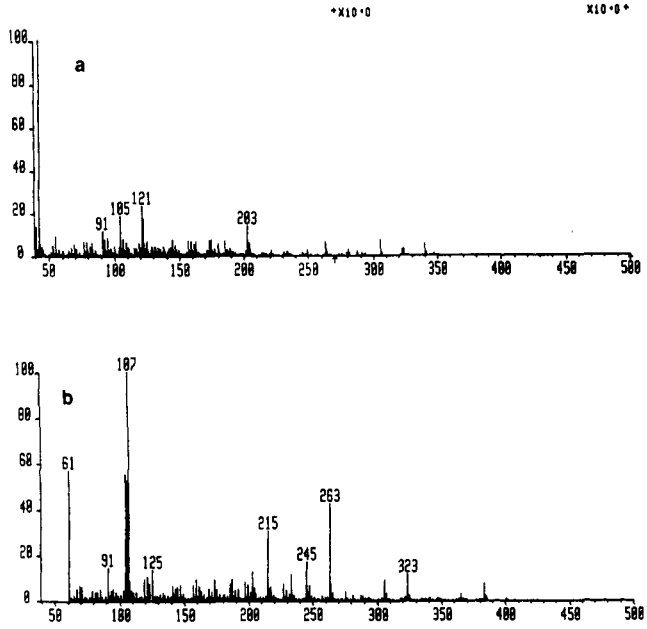


Figure 1. Probe spectra for the purified toxic metabolite: (a) electron ionization showing highest mass ion at m/z 340 (M − acetyl); (b) chemical ionization using isobutane reagent gas showing $(M + H)^+$ ion at m/z 383.

a repeller electrode at +180 V scanning from 100 to 700 μ.

Preparation of Derivatives for GC/MS Analysis. Trimethylsilyl ether derivatives were prepared by heating a small portion of extract (few micrograms) in a tapered Reacti-vial for 20 min at 60 °C together with 50 μL of BSTFA (Regis Chemicals, Phase Separation, Deeside, U.K.). Samples were analyzed directly without removing excess BSTFA reagent.

Acetylation was carried out by taking a portion of the extract (few micrograms) and heating in a tapered Reacti-vial for 1 h at 60 °C together with 1 drop of dry pyridine and 1 drop of acetic anhydride. The sample was dried with a stream of nitrogen and redissolved in ethyl acetate (50 μL) for GC/MS analysis.

RESULTS AND DISCUSSION

The toxic metabolite purified from *Fusarium compactum* cultured on a peanut substrate gave a single spot, which when visualized with 4-(*p*-nitrobenzyl)pyridine, gave the characteristics blue coloration indicative of trichothecenes. The probe EI spectrum (shown in Figure 1a) had a highest mass ion at m/a 340, which was assigned as M − 42 (loss of acetyl). Chemical ionization spectra with both isobutane and ammonia reagent gases were poor, but nevertheless, an $(M + H)^+$ ion was evident at m/z 383 (Figure 1b), confirming the proposed molecular weight as 382. Better evidence for a molecular weight of 382 was obtained by thermospray ionization in the presence of ammonium acetate (Figure 2) when an intense ion at m/z 400 $(M + NH_4)^+$ was observed, together with a discernible ion at m/z 383 $(M + H)^+$. Acetylation of the toxin followed by GC/MS analysis gave a single GC peak with a spectrum showing a molecular ion at m/z 484 (Figure 3a). This diacetyl derivative had an identical GC retention time and gave an indistinguishable spectrum to that of the product obtained by acetylation of the trichothecene neosolaniol. Trimethylsilylation of the toxin and GC/MS analysis gave a single peak and an EI spectrum with a molecular ion at m/z 526 (Figure 3b). Mass spectrometric evidence clearly showed the molecular weight of the unknown toxin to be 382 and indicated the presence of two hydroxyls as substituents together with one or more acetyl groups. By comparison with spectra of known trichothecenes and on the above evidence, together with observed fragmentations, it is proposed that the unknown toxin is isomeric with neosolaniol (I) and has the structure II.

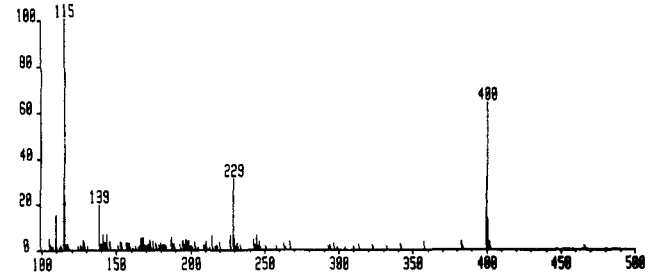


Figure 2. Thermospray ionization spectrum of the purified toxic metabolite in the presence of ammonium acetate. Highest ion observed at m/z 400 assigned as $(M + NH_4)^+$.

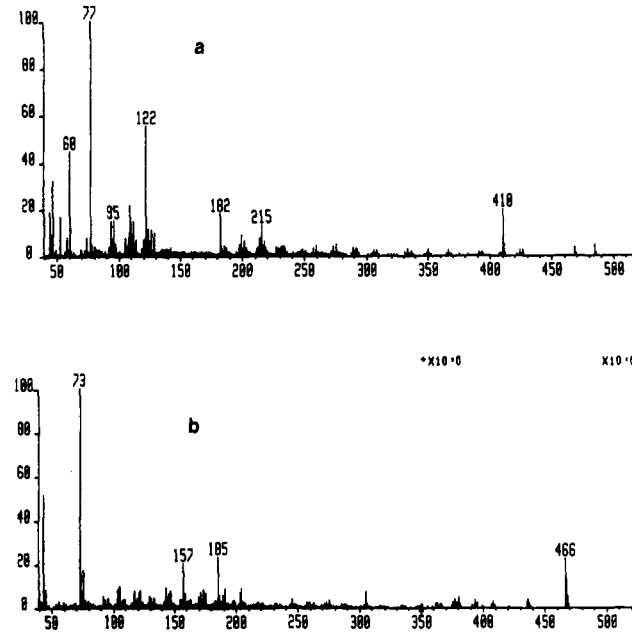
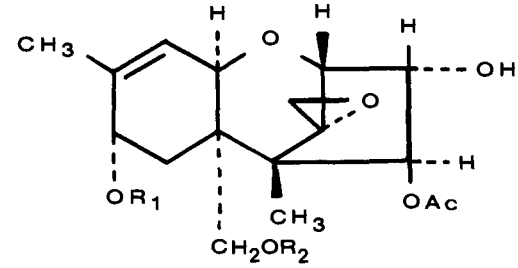


Figure 3. GS/MS spectra (electron ionization) obtained for derivatives of the purified toxic metabolite: (a) diacetyl derivative with an observed molecular ion at m/z 484; (b) bis(trimethylsilyl) derivative with an observed molecular weight of 526.



I. Neosolaniol, R_1 = H, R_2 = OAc

II. Isoneosolaniol, R_1 = OAc, R_2 = H

Proton and carbon NMR spectra support the above structure (II), in particular the OAc in the 8-position and the nonequivalence of the substituents in the 3- and 4-positions. Detailed NMR assignments are presented in Table I. Although structural assignment by mass spectrometry and NMR was carried out independently, the spectral evidence and the proposed structure have subsequently been found to be consistent with that previously

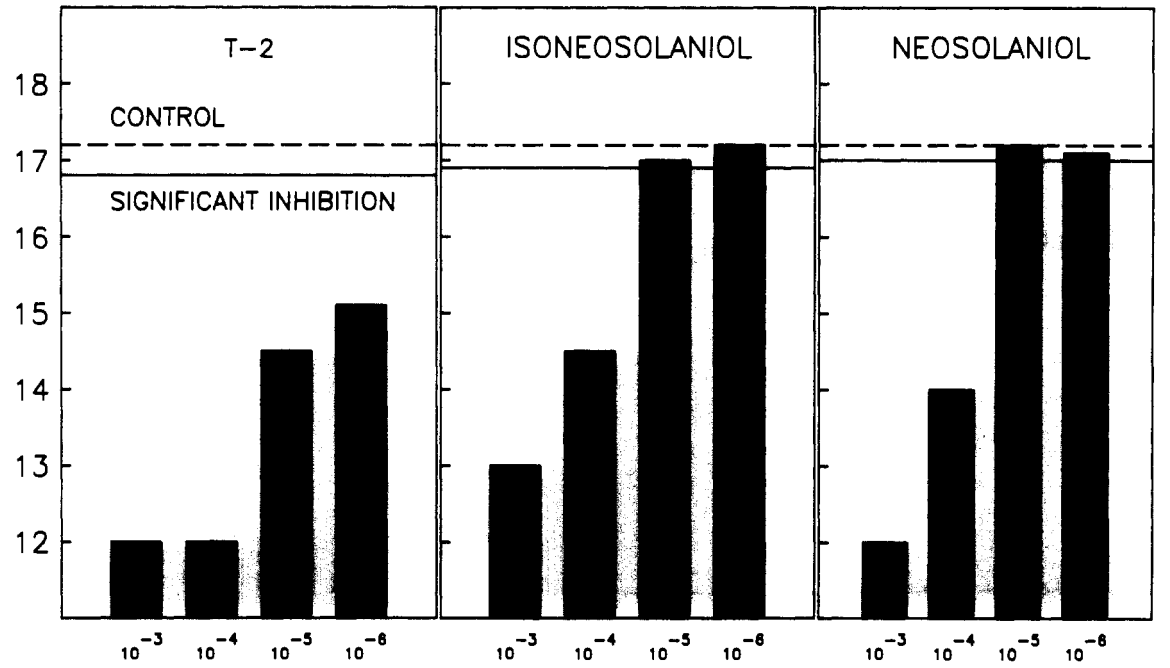


Figure 4. Plant growth regulating activity of T-2, isoneosolaniol, and neosolaniol in wheat coleoptile assays (*T. aestivum* L. cv. Wakeland): control, dotted line; significant inhibition, below solid line ($P < 0.01$).

Table I. ^1H and ^{13}C NMR Assignments (ppm) for Unknown Toxin

posn	^1H	^{13}C
2	3.60 ($J_{2,3}$ = 5 Hz)	81.3
3	4.10 ($J_{3,2}$ = 5 Hz)	79.4
4	5.31 (br s)	82.3
5		49.7
6		43.2
7	2.30 (J_{AB} = 15 Hz)	27.8
	2.04 ($J_{7,8}$ = 5 Hz)	
8	5.22 ($J_{8,7}$ = 5 Hz)	69.3
9		136.9
10	5.74 ($J_{10,11}$ = 5 Hz)	124.5
11	4.23 ($J_{11,10}$ = 5 Hz)	68.0
12		65.0
13	3.00 ($J_{A,B}$ = 4 Hz)	47.5
	2.74	
14	0.77 (s)	7.8
1k	4.28 (J_{AB} = 13 Hz)	65.3
	3.96	
16	1.72 (s)	20.9
0		
$OCCH_3$	2.02 (s)	
	2.00 (s)	21.8
C=O		171.3
OH	2.15 (s)	

reported by Ishii and Ueno (1981) for (4,8-diacetoxy-12,13-epoxytrichothec-9-ene-3,15-diol) named as NT-1 toxin. This toxin was isolated from the culture filtrate of *Fusarium sporotrichioides* together with its analogue (NT-2) containing a free hydroxyl in the 8-position.

The HEp2 cell bioassay showed the lethal concentration of isoneosolaniol to be 50–100 ng/mL. This compared to a lethal concentration of 100–200 ng/mL for T-2 toxin and 10 μg/mL for neosolaniol. Thus, the toxicity for isoneosolaniol in cell culture was far greater than neosolaniol but not too dissimilar to T-2 toxin.

In contrast, the wheat coleoptile assay showed isoneosolaniol to be less potent than T-2 or neosolaniol (Table II; Figure 4). The effect on plant growth of isoneosolaniol at 10^{-4} M was 51% inhibition compared with 100 and 61%, respectively, for T-2 and neosolaniol. Only T-2 toxin showed inhibition at 10^{-5} M with 50% inhibition of plant growth.

Table II. Percent Inhibition of Elongation by Selected Trichothecenes in Wheat Coleoptile Assay

concn, M	isoneosolaniol	neosolaniol	T-2
10^{-3}	80	100	100
10^{-4}	51	61	100
10^{-5}	0	0	50

In addition to the structural analysis of the purified metabolite subsequently shown to be isoneosolaniol, preliminary work on a less pure fraction revealed a number of characteristic trichothecene spots by TLC. GC/MS analysis of the semipure fraction showed the major peak to be isoneosolaniol, but six minor peaks were also evident, none of whose spectra were readily recognizable as known trichothecenes, with the exception of one that was indistinguishable from neosolaniol and was coincident in retention time with an authentic standard.

Workup and analysis of a sample of the waste peanuts revealed a number of trichothecene-like spots by TLC analysis, and toxicity was observed for crude fractions by the HEp2 bioassay. GC/MS analysis failed to reveal the presence of isoneosolaniol, although trace amounts of neosolaniol were detected in agreement with the findings on the cultured material.

Evidence presented elsewhere has strongly implicated waste peanuts rehydrated by snow and rain in conjunction with freezing and thawing temperatures as being involved in the intoxication of sandhill cranes in west Texas (Windingstad et al., 1987, 1988). In all cases studied, waste peanuts being used as a food source by the cranes apparently became toxic 10–14 days after rehydration. Nelson et al. (1988) have shown an overwhelming abundance of various *Fusarium* species. Of the four major species tested, *F. compactum* was the most toxigenic, with approximately 40% of the isolates producing highly toxic metabolites. The only mycotoxins detected in culture extracts of these isolates were trichothecenes, including isoneosolaniol. Other *Fusarium* species isolated from the waste peanuts are also known to produce mycotoxins; therefore, on the basis of three studies to date, the most logical cause of the sandhill crane intoxication appears to be waste peanuts contaminated with *Fusarium* spp. and

various trichothecene mycotoxins alone or in conjunction with other *Fusarium* mycotoxins.

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Comparison of Grain Composition and Nutritional Quality in Wild Barley (*Hordeum spontaneum*) and in a Standard Cultivar

Mendel Friedman* and Dan Atsmon[1]

Hordeum spontaneum (or *Hordeum vulgare ssp. spontaneum*), which is considered the wild progenitor of barley, had grain protein contents of approximately 21.5% (N × 5.7), 65% greater than that of a standard Israeli barley cultivar Ruth with 13% protein content. Additional comparative compositional studies of the two varieties revealed that the free amino acid, mineral, carbohydrate, trypsin and chymotrypsin inhibitor, and hemagglutinin (lectin) contents of the two barley genotypes differed greatly. A short-term nutritional study suggests that an equal weight of *H. spontaneum* would stimulate approximately 50% greater weight gain in mice than *H. vulgare*. *H. spontaneum* merits evaluation as a plant-breeding resource for the production of new barley varieties with high protein contents.

Natural populations of wild barley (*Hordeum spontaneum* = *Hordeum vulgare ssp. spontaneum*) in Israel contain large amounts of untapped genetic material for improving the protein contents of new varieties of barley (Nevo et al., 1985). This is an important objective since barley is the world's fourth most commonly used grain. Quality has been an important practical goal in breeding barley for brewing but not as much for animal feed, although the latter is by far the largest use of barley grain.

At present, the protein in barley cultivars is of poor nutritional quality. *H. spontaneum* has been shown (Ahokas, 1982) to have higher grain protein content than available barley cultivars, though protein contents of different accessions varies.

Since *H. spontaneum* and cultivated barley (*H. vulgare*) intercross readily and their hybrids are fully fertile, we are investigating the content and inheritance of protein and other nutrients and antinutrients in the parent genotypes and in their crosses. In this paper we report the composition and nutritional value of two potential parent genotypes. Such compositional studies provide a rational basis for breeding new barley varieties with improved nutritional and agronomic properties.

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MATERIALS AND METHODS

Trypsin, chymotrypsin, benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), *N*-acetyl-L-tyrosine ethyl ester (ATEE), and the other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

The locally bred cultivar Ruth is two-rowed high-yielding barley with ca. 50-mg grain weight and 12% protein in the grains, under normal field conditions. The *H. spontaneum* line was an advanced selfed generation of accession No. 297 from a collection supplied by Dr. E. Nevo (Haifa University) with 35–40-mg grain weight and 25% protein in the grain under normal conditions. Seeds of both varieties for the detailed analyses were collected from the same environment (neighboring field plots) and received identical water and nutritional regimens. The *H. spontaneum* brittle heads were manually threshed, and seeds were partially peeled to remove extra rachis and glume parts. These parts were removed from the cultivar seeds by ordinary threshing.

Proximate Analyses. Kjeldahl nitrogen, moisture, carbohydrate, fat, fiber, and mineral contents were measured by standard techniques (AOAC, 1980).

Amino Acid Analyses. Amino acid analyses were carried out with about 5 mg of protein hydrolyzed in evacuated tubes with 6 N HCl for 24 h at 110 °C. The hydrolysates were then evacuated to dryness with the aid of a water aspirator. Aliquots of the dry residue dissolved in pH 2.2 citrate buffer were analyzed on a Model D 500 Durrum amino acid analyzer under the following condi-

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