30 min and development at alkaline pH. The sp. act. was computed based on a standard definition that 1 unit of enzyme activity produces 1.0 μmol p-nitrophenol per min at 25°. Protein determinations were by Lowry [14]. MWs were determined by gel filtration on a column 1×100 cm packed with Sephadex G-200 equilibrated with 0.05 M Tris–HCl at pH 7.5. A series of standard proteins were also run to calibrate the column which was continuously monitored. The metal ion content of the proteins was determined by Galbraith Laboratories, Knoxville, TN using atomic absorption spectrophotometry. The 3-dimensional structure of the molecule was determined by conventional X-ray diffraction techniques using isomorphous replacement with 5 heavy atom derivatives. The details of this analysis will be published elsewhere [5].

REFERENCES

1. Sumner, J. B. (1919) *J. Biol. Chem.* **37,** 137.
2. Sumner, J. B. and Howell, S. F. (1936) *J. Biol. Chem.* **113,** 607.
3. Goldstein, I. J., Hollerman, C. E. and Smith, E. E. (1965) *Biochemistry* **4,** 876.
4. Callow, J. A. (1975) *Curr. Adv. Plant Sci.* **7,** 181.
5. Smith, S. C. and McPherson, A. (1980) *J. Biol. Chem.* (in press).
6. McPherson, A. (1980) *J. Biol. Chem.* (in press).
7. Li, Y.-T. and Li, S.-C. (1972) *Methods Enzymol.* **28,** 702.
8. Li, Y.-T. (1967) *J. Biol. Chem.* **242,** 5474.
9. Snaith, S. M. and Levvy, G. A. (1968) *Biochem. J.* **110,** 663.
10. Snaith, S. M. (1975) *Biochem. J.* **157,** 83.
11. McPherson, A. and Spencer, R. (1975) *Arch. Biochem. Biophys.* **169,** 650.
12. Laemmli, U. K. (1970) *Nature* **227,** 680.
13. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.

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N-BENZOYLASPARTATE AND *N*-PHENYLACETYLASPARTATE FROM PEA SEEDS*

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INTRODUCTION

In our continuing study of the metabolism of developing fruits and seeds of the G2 line of peas, we have isolated and identified two naturally occurring *N*-acyl amino acids which have not been reported previously. These compounds are the aromatic amides of aspartic acid, namely, *N*-benzoylaspartate and its homologue *N*-phenylacetylaspartate.

A variety of acyl amino acids have been found in living systems; *N*-acetylaspartate is present in mammalian brain tissue [1], hippuric acid (*N*-benzoylglycine) is a normal constituent of human urine [2] and *N*-phenylacetylglutamine has been found in cow's milk [3]. In plants, the metabolism of exogenously supplied benzyl alcohol and benzoic acid to *N*-benzoylaspartate has been reported in barley [4], and in auxin-treated pea stem segments [5]. The following paper describes the isolation and identification of these substances by chemical tests and MS analysis. The results were confirmed by MS of the chemically synthesized derivatives.

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RESULTS AND DISCUSSION

Developing fruits were enclosed in glass chambers in the presence of $^{14}CO_2$ for 24 hr. The acidic EtOAc-soluble fraction of the 80% MeOH extract was chromatographed by Si gel HPLC in $CHCl_3$–MeOH–HOAc (70:30:1). The radioactive zone co-chromatographing with abscisic acid (ABA) was removed and chromatographed in $CHCl_3$–MeOH–HOAc (90:10:1). The major radioactive zone (R_f 0.125) was clearly separated from ABA (R_f 0.46) in this system.

Chemical tests for functional groups indicated that

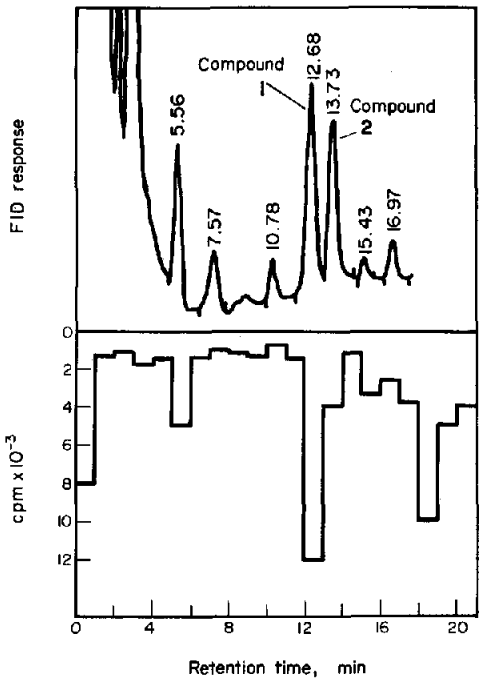

Fig. 1. GC–RC trace of compounds **1** and **2**.

this labelled material was a carboxylic acid (positive reaction with CH_2N_2), without a hydroxyl group (negative reaction with Ac_2O), or, ketone or aldehyde groups (negative reaction with 2,4-dinitrophenylhydrazine).

GC–RC of the methylated material split the radioactivity into 4 fractions (Fig. 1). GC and GC–MS of a nonlabelled seed extract similarly purified indicated the presence of several fatty acid esters and two unknown compounds (which had similar R_fs to one of the labelled zones from GC–RC) with the following MS (*m/e* (%)): (compound **1**) GC–MS (70 eV) 265(0.9), 234(0.7), 207(3.9), 206(32.8), 174(2.7), 160(7.2), 146(1.1), 128(1.3), 113(2.6), 105(100), 77(69.2), 51(10.0), 43(1.1), and (compound **2**) GC–MS (70 eV), 280(0.8), 279(5.2), 248(1.6), 220(18.3), 188(22.7), 160(58.1), 156(18.6), 146(3.0), 128(44.8), 118(20.3), 113(24.4), 102(91.6), 91(100), 86(42.0), 65(22.6), 59(14.8), 43(5.8).

Chemical ionization (CI) GC–MS of compound **1** confirmed the assumption that *m/e* 265 was the M^+: (CI)GC–MS 266[$M^+ + 1$](79.8), 234[$M^+ - 31$](53.0), 206[$M^+ - 59$](34.9). The M^+ is at an odd mass indicating the presence of N. Peaks at *m/e* 234 and 206 are associated with the loss of the Me/ester group. Elemental composition data for the *m/e* 206 ion indicate a maximum of 11 carbons (A+1/A = 11.89%) and, therefore, 13 carbons for the molecule. Additional ions at *m/e* 174 and 146 could result from the loss of 32 and 60 amu from the *m/e* 206 ion due to the presence of a second Me ester group. The base peak at *m/e* 105 is probably the benzoyl cation.

(CI)GC–MS of compound **2** confirmed the assignment of *m/e* 279 as the M^+: *m/e* 280 [$M^+ + 1$](22.7). The M^+ is at an odd mass indicating N is present. The carbon isotopes ratio for the M^+ predicts 14 carbon atoms (M+1/M = 15.38%). Peaks at *m/e* 248 [$M^+ - 31$] and *m/e* 220 [$M^+ - 59$] indicate a Me ester group. Additional ions at *m/e* 188 and 160 could result from cleavage of a second Me ester group with hydrogen rearrangement from the *m/e* 220 fragment. The base peak at *m/e* 91 is most likely the benzyl or tropylium ion. Compound **2** has a MW 14 amu higher than **1** and is without an ion at *m/e* 105. In this molecule a methylene group could be inserted between phenyl and the carbonyl carbon. Ions at *m/e* 118, 102, and 43 can be rationalized if an amide group is present. *M/e* 118 could be the phenylketene cation resulting from cleavage of the carbonyl carbon–nitrogen bond with hydrogen rearrangement to the N atom. The *m/e* 102 peak could be due to cleavage of a carbon–carbon bond beta to the N atom with a loss of 59 amu, followed by cleavage of the carbonyl carbon–nitrogen bond accompanied by the rearrangement of H to the N containing fragment with loss of phenylketene. The ion at *m/e* 43 is found in many secondary amides [6], although CONH is not the only possible structure for this fragment.

On the basis of these data and the chemical tests, the most likely structure for compound **1** is: *N*-benzoylaspartate and for compound **2**: *N*-phenylacetylaspartate. These structures were confirmed by chemical synthesis of the compounds from aspartic acid and either benzoylchloride or phenylacetylchloride. The MS of the synthetic compounds were identical to those derived from the seed extract. Acid hydrolysis of the synthetic compounds gave 2 spots after TLC, one ninhydrin positive, cochromatographing with aspartic acid, and the other cochromatographing with either benzoic acid or phenylacetic acid, respectively [7].

The possible pathways leading to the synthesis of *N*-benzoylaspartate and *N*-phenylacetylaspartate could involve the catabolism of phenylalanine to phenylacetic acid and/or benzoic acid, followed by condensation with aspartic acid to form the amides [8, 9]. Hippuric acid-[^{14}C] has been isolated from humans previously fed phenylalanine-[^{14}C]; all of the label was found in benzoate after acid hydrolysis [10]. The synthetic pathways of these compounds in peas are being studied to determine whether they conform with the above scheme.

We do not know the function of these compounds in the plant, although some role in the promotion of plant senescence or the inhibition of growth and development, including seed germination, is a possibility. Working with a series of synthetic *N*-acyl amino acids, Omari *et al.* [11] found that *N*-benzoylaspartate was the only *N*-benzoyl amino acid to significantly inhibit the germination of rice and radish seeds. We have tested *N*-benzoylaspartate and *N*-phenylacetylaspartate as inhibitors of both mature and precociously germinated pea seeds and noted a slight reduction in radicle growth at 20 mM.

EXPERIMENTAL

Plant extraction. Developing pea seeds (100 g) were homogenized in 80% MeOH. The aq. soln remaining after evapn was made basic (pH 9) and partitioned against hexane. The aq. fraction was acidified to pH 3 and partitioned against EtOAc.

TLC. The EtOAc fraction was chromatographed by PLC on 1 mm Si gel H (washed and run in MeOH before use) with $CHCl_3$–MeOH–HOAc (70:30:1). R_f zone 0.33–0.67 was

removed and eluted with MeOH. The eluate was centrifuged, filtered through millipore (membrane type) and methylated with CH_2N_2–Et_2O. The methylated extract was chromatographed by PLC with $CHCl_3$–EtOAc (1:1) and R_f zone 0.67–0.92 eluted with EtOAc.

GC and GC–MS. Samples from PLC were chromatographed on silanized glass columns 1.8 m × 2 mm packed with 2% OV-17. Operating conditions were: 175° isothermal for 6 min, temp. programmed to 250° at 4°/min with He at 29 ml/min. Injection temp. was held at 250° and FID detector at 300°.

GC–RC. The effluent from the column was divided by an all glass outlet splitter to a heated collection vent held at 200°. Samples were collected at room temp. in capillary tubes packed with 2 cm of 2% OV-17. The radioactivity was washed out with MeOH into scintillation vials and counted. All metal fittings and glass tubing in the GC system were coated with SE-30 liquid phase.

Chemical tests. Reaction with CH_2N_2: the radiolabelled sample was dissolved in a minimal vol. of MeOH and CH_2N_2–Et_2O added dropwise until a yellow color persisted for 1 hr. Acetylation: reaction with Ac_2O–Py (1:1) for 24 hr at 20° in the dark. Carbonyl test: reaction with 2,4-dinitrophenylhydrazine (0.15%) in HOAc for 24 hr at 20° in the dark. A shift in R_f after TLC was considered indicative of a positive reaction.

Synthesis of **1**. Schotten–Baumann reaction [12]. The product was boiled in CCl_4 and recrystallized from Me_2CO–hexane.

Synthesis of **2**. The aminodiester HCl was first synthesized by reacting aspartic acid (10 g) with $SOCl_2$ in MeOH for 1 hr under reflux. The product was suspended after evapn in aq. Na_2CO_3 at 4° and extracted into EtOAc. The aminodiester was acylated with phenylacetylchloride in $(C_2H_5)_3N$. The ppt. was extracted into EtOAc, dried and dissolved in MeOH. The ester groups were hydrolysed by the addition of 0.2 N NaOH to a pH of 10 for 24 hr at 30°. The product was purified by PLC in $CHCl_3$–MeOH–HOAc (70:30:1).

Acid hydrolysis of amides. **1** and **2** were hydrolysed in dil mineral acid at 100° for 12 hr. The products were chromatographed in C_6H_6–MeOH (19:1) and *n*-BuOH–HOAc–H_2O (4:1:1).

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REFERENCES

1. Tallan, H. H., Moore, S. and Stein, W. H. (1956) *J. Biol. Chem.* **219,** 257.
2. Stein, W. H., Paladini, A. C., Hirs, C. H. W. and Moore, S. (1954) *J. Am. Chem. Soc.* **76,** 2848.
3. Schwartz, D. P. and Pallansch, M. J. (1962) *Nature* **194,** 186.
4. June, J. A., Prem, S. and Gholson, R. K. (1976) *Phytochemistry* **15,** 647.
5. Venis, M. A. and Stoessl, A. (1969) *Biochem. Biophys. Res. Commun.* **36,** 54.
6. Gilpin, J. A. (1959) *Analyt. Chem.* **31,** 935.
7. Capindale, J. P. and Fan, H. S. (1967) *Can. J. Chem.* **45,** 1921.
8. Meister, A. (1965) *Biochemistry of the Amino Acids,* Vol. 2, p. 929. Academic Press, New York.
9. Pitt, B. M. (1962) *Nature* **196,** 272.
10. Grümer, H. D. (1961) *Nature* **189,** 63.
11. Omari, M., Hasegaua, T., Suzuki, T. and Sahashi, Y. (1972) *Mem. Tokyo Univ. Agric.* **15,** 31.
12. Greenstein, J. P. and Winitz, M. (1961) *Chemistry of the Amino Acids,* Vol. 2, p. 1266. John Wiley, New York.