Rico, were extracted continuously with $CHCl_3$ for 3 days and worked up in the usual fashion [9]. TLC of the crude gum (5 g) indicated the presence of a large number of relatively non-polar substances, each in rather small amount, which could not be characterized satisfactorily. Extraction of the roots (250 g) gave 3.5 g of crude gum which was absorbed on 5 g silicic acid (Mallinckrodt, 100 mesh) and chromatographed over 200 g of the same absorbent packed in hexane, 250 ml fractions being collected as follows: 1–2 (hexane), 3–6 (hexane–EtOAc, 19:1) 7–10 (hexane–EtOAc, 9:1), 11–14 (hexane–EtOAc, 4:1), 15–18 (hexane–EtOAc, 3:2), 19–22 (hexane–EtOAc, 2:3), 23–26 (hexane–EtOAc, 1:4), 27–28 (EtOAc), 29–30 (EtOAc–MeOH, 19:1) and 31–32 (EtOAc–MeOH, 9:1). Fraction 8 upon purification by TLC (C_6H_6–EtOAc, 9:1) gave 15 mg costunolide. Fraction 9 on standing in hexane–EtOAc deposited 22 mg of a mixture of sitosterol and stigmasterol. Fraction 12 showed two spots; separation by prep. TLC (C_6H_6–EtOAc, 9:1) yielded 70 mg of a mixture of linolenic and stearic acids as the less polar material. The lower band on purification by TLC (7% $AgNO_3$–silica gel, C_6H_6–EtOAc, 9:1, several developments) furnished the unstable amides **2** (22 mg) and **3** (24 mg). ^1H NMR spectral data are reported in Table 1. Amide **3** had IR bands ($CHCl_3$ at 3400 br, 1670 and 1670 cm^{-1}); the low-resolution MS exhibited peaks at *m/z* (rel. int.): 247 $[M]^+$ (1.9), 167 (25.5) and 81 (100).

Acknowledgements—We wish to thank Mr. César Xavier Hernandez for the collection and Dr. José Hernandez for bringing the problem to our attention.

REFERENCES

1. Garcia Barriga, H. (1975) *Flora Medicinal de Colombia Botanica Medica*, Vol. 3, p. 396. Instituto de Ciencias Naturales, Universidad Nacional, Bogota, Colombia.
2. Nash, D. L. and Williams, L. O. (1976) *Flora of Guatemala, Fieldiana: Botany*, Vol. 24, Part XII, p. 301.
3. Bohlmann, F., Ziesche, J., Robinson, H. and King, R. M. (1980) *Phytochemistry* **19**, 1535.
4. Jacobson, M. (1948) *J. Am. Chem. Soc.* **70**, 4234.
5. Jacobson, M. (1949) *J. Am. Chem. Soc.* **71**, 366.
6. Jacobson, M. (1967) *J Org. Chem.* **32**, 1646.
7. Hartman, R. L. and Stuessy, T. F. (1983) *Syst. Botany* **8**, 185.
8. Robinson, H. (1981) *Smithsonian Contrib. Botany* **51**, 1.
9. Herz, W. and Högenauer, G. (1962) *J. Org. Chem.* **27**, 905.

Phytochemistry, Vol. 24, No 1, pp. 174–176, 1985.
Printed in Great Britain.

0031-9422/85 $3.00 + 0.00

IDENTIFICATION AND DISTRIBUTION OF ONONITOL IN NODULES OF *PISUM SATIVUM* AND *GLYCINE MAX*

JOHN G. STREETER

Department of Agronomy, Ohio State University, Wooster, OH 44691, U.S.A.

(*Received* 30 *March* 1984)

Key Word Index—*Pisum sativum*; *Glycine max*; Leguminosae; pea; soybean; cyclitol; 4-*O*-methyl-*myo*-inositol.

Abstract—Ononitol (4-*O*-methyl-*myo*-inositol) was identified as a major carbohydrate in *Pisum sativum* nodules, comprising 25–34% of the total mono- plus disaccharides in nodules formed by two *Rhizobium leguminosarum* strains. Ononitol was purified from *Glycine max* nodules and was found to be a minor carbohydrate in these nodules. The distribution of ononitol in bacteroids and cytosol from soybean nodules suggests that it is not synthesized by bacteroids.

In the analysis of TMSi derivatives of carbohydrates from pea (*Pisum sativum*) nodules by GC, a major peak was found with a retention time very similar to that of sequoyitol (5-*O*-methyl-*myo*-inositol) using a column of 3% OV-17 and a temperature programme of 150° (8 min hold) increasing at 5°/min to 240°. However, the retention time of the unknown, relative to penta-TMSi-β-phenyl-glucose (internal standard), was consistently 0.02 units different from the RR_t of TMSi sequoyitol.

The unknown was a neutral compound as indicated by its failure to bind to columns of Dowex 50-H^+ or Dowex 1-formate. The unknown was hydrolysed only under harsh conditions (3 M HCl, 105° for several hr). Hydrolysis for increasing periods of time (1–13 hr) gave increasing amounts of a compound, the TMSi derivative of which co-chromatographed precisely with TMSi *myo*-inositol on OV-17. The decrease in unknown was proportional to the increase in *myo*-inositol.

The neutrality and remarkable stability of the unknown and the hydrolytic conversion of the unknown to *myo*-inositol indicated that the compound was an *O*-methyl-cyclitol in the *myo*-inositol family. Only three *O*-methyl

derivatives of *myo*-inositol are known to occur in nature: sequoyitol, bornesitol (1-*O*-methyl-*myo*-inositol) and ononitol (4-*O*-methyl-*myo*-inositol) [1–3]. Authentic bornesitol was easily separated from the unknown cyclitol in pea nodules under the conditions described above. However, sequoyitol and ononitol could not be separated either as TMSi or acetyl derivatives on packed columns of 3% OV-17, 3% SP-2100, or 5% SE-52 using several different temperature programmes with each column.

As recently reported by Binder and Hadden [2], sequoyitol and ononitol were separated on a 2% Silar 10C column. When a temperature programme of 145° (10 min) increasing at 10°/min to 245° was used, the retention times of TMSi ononitol and TMSi sequoyitol were 12.06 and 12.22 min, respectively. When a programme of 180° (5 min) increasing at 10°/min to 245° was used, the retention times of penta-*O*-acetyl ononitol and penta-*O*-acetyl sequoyitol were 12.46 and 13.39 min, respectively. Under these conditions, the unknown cyclitol from pea nodules had a retention time identical to that of ononitol. When authentic TMSi or acetyl ononitol was added to the samples, a peak without shoulders having the retention time of ononitol resulted.

I previously identified D-pinitol (3-*O*-methyl-*chiro*-inositol), D-*chiro*-inositol, and *myo*-inositol as major carbohydrates in soybean nodules [4, 5] and suggested that sequoyitol was a minor cyclitol present in soybean nodules [4]. In attempting to confirm this identification using the Silar 10C column it was found that *chiro*-inositol chromatographs very close to the minor cyclitol and, because of the very large amount of *chiro*-inositol present, a clean separation could not be achieved.

A small amount ($\leq$ 10 mg) of the minor cyclitol was purified from *ca* 1 kg of soybean nodules. The product, which appeared > 99% pure based on GC analysis, co-chromatographed with ononitol using the Silar 10C column and the conditions described for analysis of the pea nodule cyclitol. It was concluded that ononitol is a major carbohydrate in pea nodules and a minor carbohydrate in soybean nodules. Thus, previous reports [4, 6] suggesting the presence of sequoyitol in soybean nodules are incorrect.

Ononitol comprised 92–97% of the total cyclitols and 25–34% of the total mono- plus disaccharides in pea nodules formed by two strains of *Rhizobium leguminosarum* (Table 1). In contrast, ononitol comprised only 9% of the total cyclitols and 4% of total mono- plus disaccharides in soybean nodules grown at the same time and under the same conditions.

In the analysis of relative concentration of carbohydrates in bacteroids and cytosol from soybean nodules we find three different patterns (Fig. 1): (a) α,α-trehalose, which is synthesized in bacteroids [7] and which is largely retained by bacteroids; (b) compounds like *myo*-inositol which are apparently synthesized in bacteroids but which are recovered largely in the cytosol; (c) compounds like ononitol which are apparently synthesized in plant parts other than nodules. Compounds in class (b), which includes *chiro*-inositol, have concentrations in nodules 7- to 10-fold greater than other plant parts [4] and show declining concentrations in bacteroids in experiments like that illustrated in Fig. 1. Compounds in class (c), which includes pinitol, sucrose, glucose and fructose, have concentrations in nodules which are similar to or less than concentrations in other plant parts [4] and low and constant concentrations in bacteroids in experiments like that illustrated in Fig. 1. Thus, while the difference in ononitol concentration in pea nodules formed by different *Rhizobium leguminosarum* strains (Table 1) might be interpreted to indicate synthesis in bacteroids, the distribution of ononitol in soybean nodules is very similar to that of sucrose or pinitol and this distribution pattern does not indicate synthesis of ononitol in bacteroids.

While ononitol has long been known as a constituent of higher plants, there are no reports, to my knowledge, regarding the biosynthesis of ononitol. Ononitol has recently been reported to be a probable precursor of D-pinitol in *Simmondsia chinensis* [8]. Galatosyl ononitol has recently been reported as a constituent of the seeds of *Vigna angularis* [9] and ononitol is apparently widely distributed among legumes [3]. (D-(+)-Ononitol is the only isomer reported to occur in nature [1]). The role of cyclitols in legume nodules is unknown. In soybean nodules, none of the cyclitols is readily labelled by incoming [^{14}C]photosynthate [7]. Perhaps the cyclitols play some osmotic role as has recently been suggested for pinitol in *Sesbania aculeata* [10], and also for ononitol, a major carbohydrate in most *Vigna* species [11].

EXPERIMENTAL

Plant material. Seeds of soybean, *Glycine max* cv Beeson and pea, *Pisum sativum* cv Alaska were planted in pots containing

Table 1. Concentration of cyclitols in root nodules of *Pisum sativum* and *Glycine max*

Legume	Rhizobium strain	Cyclitol	Concn* (mg/g fr. wt)	% of total cyclitol	% of total monosaccharide + disaccharide†
Pisum sativum	128C53	Ononitol	3.23 (0.31)	97	34
		myo-Inositol	0.10 (0.01)	3	<1
Pisum sativum	128C78	Ononitol	1.37 (0.32)	92	25
		myo-Inositol	0.12 (0.01)	8	<1
Glycine max	NR-6‡	Pinitol	0.55 (0.01)	24	11
		chiro-Inositol	0.62 (0.02)	27	12
		myo-Inositol	0.90 (0.04)	40	18
		Ononitol	0.19 (0.01)	9	4

*Mean, and s.e. in parentheses, of 3 replicates.

†Includes fructose, glucose, sucrose, α,α-trehalose, maltose and the cyclitols shown.

‡Derivative of 61A76.

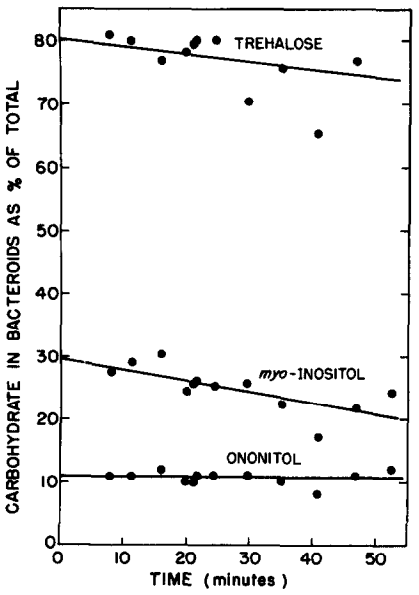

Fig. 1. Proportional concentrations of carbohydrates in bacteroids as a function of time after grinding soybean nodules. Nodules were formed by *Rhizobium japonicum* USDA 110. Nodules were ground in water (1°) and the mixture was filtered through four layers of cheesecloth. The extract was kept at 1° for varying periods of time before centrifugation at 27 000 *g* for 2 min. Bacteroids and cytosol were mixed with hot (80°) 95% ethanol and, after extraction of bacteroid metabolites, carbohydrate concentration was determined and expressed as % of total compound in bacteroid + cytosol fractions. For α,α-trehalose and ononitol, there was a small decline (6%) in the total amount of compound recovered during the time period shown (first three samples vs last three samples). For *myo*-inositol, there was no decline in the total amount of compound during the period shown. The mean concentrations (across all times) of trehalose, *myo*-inositol and ononitol in bacteroids were 126, 271 and 23 μg/g fr. wt, respectively. The lines shown are the calculated regression lines.

14 kg of washed, autoclaved silica sand. Seeds were inoculated with liquid cultures of *Rhizobium leguminosarum* or *R. japonicum* and plants were irrigated twice daily with a nutrient solution lacking combined N [7]. Nodules were obtained from plants 35–55 days after planting.

Extraction and analysis. Whole nodules were ground with 95% EtOH and, after centrifugation, the solid residue was extracted 3 times with 75% EtOH. Combined extracts were dried *in vacuo* and solids were dissolved in H_2O and stored with a few drops of $CHCl_3$ at 1°. Aliquots of the aq. soln were dried in small vials with a stream of air.

Solids were dissolved in pyridine and TMSi derivatives formed using HMDS (200 μl) + TFA (20 μl) (30 min, room temp.). Acetyl derivatives were formed by dissolving solids in Ac_2O (200 μl) + TFA (20 μl) and heating (70°) for 30 min. Acetyl derivatives were dried under N_2 at 50° and redissolved in pyridine for injection into the GC.

Columns of 3% OV-17, 3% SP 2100, and 5% SE-52 were 1830 × 2 mm and the 2% Silar 10C column was 3050 × 2 mm. Injection and detector (FID) temps were 285 and 330°, respectively, and the carrier gas was He at a flow rate of 30 ml/min.

Purification of ononitol. Soybean nodules were extracted with EtOH as described above. The aq. extract was passed through tandem 2 × 20 cm columns of Dowex 50 (H^+) and Dowex 1 (OH^-) which remove sugars as well as all ionizable compounds [6]. Column effluent was dried *in vacuo*; solids were dissolved in water and streaked on S & S 2043b paper. Papers were irrigated (descending) with Me_2CO–H_2O (17:3) [12] for 13 hr. Papers were dried and cyclitols were located on 2 cm strips on both edges of the paper using $AgNO_3$ [12]. The ononitol streak was eluted from the center of the paper with water. Ononitol was contaminated (*ca* 10%) with D-*chiro*-inositol, but purity ⩾ 99% could be obtained by re-chromatographing the ononitol using the same system.

Acknowledgements—I thank L. Anderson and O. Hoffmann-Ostenhof for samples of sequoyitol, L. Anderson for a sample of bornesitol, S. J. Angyal for a sample of penta-*O*-acetyl-ononitol, P. Wong for *Rhizobium leguminosarum* strains, C. A. Neyra for *R. japonicum* strain NR-6, and Paul DeVine and Mary Kilpatrick for technical assistance. Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article 122-84.

REFERENCES

1. Angyal, S. J. and Anderson, L. (1954) *Adv. Carbohydr. Chem.* **14**, 135.
2. Binder, R. G. and Hadden, W. F. (1984) *J. Agric. Food Chem.* **32**, 685.
3. Kindl, H. and Hoffmann-Ostenhof, O. (1966) *Prog. Chem. Org. Nat. Prod.* **24**, 149.
4. Streeter, J. G. (1980) *Plant Physiol.* **66**, 471.
5. Streeter, J. G. and Bosler, M. E. (1976) *Plant Sci. Letters* **7**, 321.
6. Phillips, D. V., Dougherty, D. E. and Smith, A. E. (1982) *J. Agric. Food Chem.* **30**, 456.
7. Reibach, P. H. and Streeter, J. G. (1983) *Plant Physiol.* **72**, 634.
8. Dittrich, P. and Korak, A. (1984) *Phytochemistry* **23**, 65.
9. Yasui, T. (1980) *Agric. Biol. Chem.* **44**, 2253.
10. Gorham, J., McDonnell, E. and Wyn Jones, R. G. (1984) *Z. Pflanzenphysiol.* **114**, 173.
11. Ford, C. N. (1982) *Phytochemistry* **21**, 1149.
12. Kindl, H. and Hoffmann-Ostenhof, O. (1966) *Phytochemistry* **5**, 1091