Phytochemistry, Vol. 20, No. 5, pp. 1125–1127, 1981.
Printed in Great Britain.

0031–9422/81/051125–03 $02.00/0

SHORT REPORTS

OCCURRENCE, STRUCTURE AND SYNTHESIS OF 3-(*N*-METHYLAMINO)GLUTARIC ACID, AN AMINO ACID FROM *PROCHLORON DIDEMNII*

ROGER E. SUMMONS

Research School of Biological Sciences, Australian National University, Canberra, ACT, Australia

(*Received* 29 *August* 1980)

Key Word Index—*Prochloron didemnii*; didemnid ascidans; amino acid structure determination; 3-(*N*-methylamino)glutaric acid.

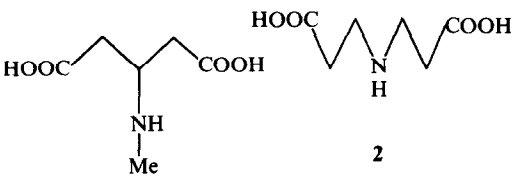
Abstract—3-(*N*-Methylamino)glutaric acid has been identified as a new free amino acid in extracts from *Prochloron didemnii* (Lewin), a unique prokaryotic algal symbiont associated with certain didemnid ascidians. Its structure was established by elucidation of the mass spectra of its TMSi and other derivatives and confirmed by synthesis.

INTRODUCTION

Prochloron didemnii is a unique unicellular alga, being prokaryotic, having a chlorophyll composition normally associated with green plants, and living in an exosymbiotic association with certain primitive chordates of the family Didemnidae [1–4]. Although electron microscopy reveals differences between the alga isolated from different didemnids [1], the genus *Prochloron* is currently recognized as being monospecific. Evidence has been presented for placing *Prochloron* in a new algal division (Prochlorophyta) [5] and also for its inclusion in the Cyanophyta [6, 7]. In the course of experiments to determine some of the characteristics of nitrogen assimilation in the symbiont of *Didemnum molle*, an unusual and major component was consistently observed in the acidic amino acid fraction isolated from the alga.

RESULTS AND DISCUSSION

GC/MS analysis of TMSi derivatives of acidic amino acid fractions from extracts of *Prochloron* from *D. molle* revealed a peak *ca* 5 times the magnitude of and eluting slightly after glutamic acid. The EI mass spectrum of the compound showed a M^+ at *m/z* 377 and indicated the presence of three TMSi groups. DiMe ester and *N*-trifluoroacetyl dibutyl ester (TAB) derivatives were prepared and the M^+ of the latter compound was accurately measured to establish an elemental composition of $C_6H_{11}NO_4$ for the natural product. In the spectrum of the TMSi derivative there appeared to be only one major α-cleavage fragmentation giving rise to the base peak at *m/z* 246 ($M^+ - 131$) suggesting that the molecule had a plane of symmetry passing through the nitrogen atom. Hence loss of a $\cdot CH_2$–COOTMSi radical from either side resulted in a common charged fragment. Since only two simple structures (**1** and **2**) were likely candidates both were synthesized.



1 **2**

3,3′-Iminodipropionic acid **2** was prepared by Michael addition of ammonia in anhydrous ethanol to ethyl acrylate [8] and mass spectra of derivatives of this compound were different from those of the natural product. 3-(*N*-Methylamino)glutaric acid (**1**) was synthesized by modification of the procedure used for preparation of the *N*-nor analogue [9] which, incidentally, was also first isolated from a marine source (*Chondria armata*, Rhodophyceae). Mass spectra of the TMSi, diMe ester and TAB derivatives of the synthetic compound and their GC R_t data confirmed its identity with the natural product. The synthetic and natural products behaved identically in several TLC systems and both gave only a very faint yellow colour reaction with ninhydrin.

Since uncommon molecules such as this may be useful as taxonomic markers, evidence for its presence in the algae from other species of didemnids was sought. Results of these experiments are shown in Table 1 where the amounts of 3-(*N*-methylamino)glutaric acid are expressed semiquantitatively as a percentage of the glutamate content. The large differences in its relative abundance in a number of hosts suggests that algae may vary biochemically over the host range.

EXPERIMENTAL

Plant material. Colonies of *Didemnum molle* were collected at low tide from reef flat areas at Lizard Island (14°, 40″ S, 145° 28″ E) on Australia's Barrier Reef, and freeze-dried, preserved

Table 1. Occurrence of 3-(*N*-methylamino)glutaric acid in *Prochloron* from various didemnid hosts

Host species/source	Parts extracted	% Abundance relative to glutamate
Didemnum molle Lizard Island	Washed algal cells	500
D. molle Palau D.G. PRO-1	Algal cells + mucous	500
Trididemnum sp. Palau R.A.L. 79-T	Washed algal cells	300
Trididemnum sp. Palau R.A.L. 79-B	Host 'blood' with algal cells removed	50
Lissoclinum patella Palau R.A.L. 79-L	Washed algal cells	10
Lissoclinum sp. Fiji R.A.L. 78-4 and R.A.L. 78-8	Whole colonies host + alga	2
Trididemnum solidum St. Croix, U.S.V.I. R.A.L. 79-2	Whole colonies host + alga	Not detected
Didemnum moselyi Heron Is., G.B.R. R.A.L. 76-17	Whole didemnids, no algal cells in evidence	Not detected
Diplosoma virens Hawaii, R.A.L. 76-1	Algal cells expressed from host	2–5

specimens identified by Patricia Mather, Queensland Museum. Alga isolated from *D. molle* collected at Palau and preserved in EtOH was supplied by Professor D. Griffiths, James Cook University, Townsville. Freeze-dried and EtOH-preserved specimens of all other algae and intact colonies were supplied by Professor R. A. Lewin, Scripps Institution of Oceanography, La Jolla, California.

Isolation of alga from colonies of D. molle. Several methods of isolation were employed in order to minimize contamination by host metabolites. Colonies were dissected through the central cloacum and algal cells freed from their gelatinous mucous matrix by brief contact with 50% EtOH and immediate dilution with H_2O. Algal cells were centrifuged and the supernatant was discarded. The algal pellet was then washed once with H_2O and extracted with a mixture of EtOH–HOAc–H_2O (8:1:2). *D. molle* from Palau was found to liberate algal cells, with mucous, by gentle agitation of intact colonies. These cells were also extracted with the above solvent. Alga from several collections of *Lissoclinum patella* and *Diplosoma virens* were expressed by squeezing, centrifuged and washed prior to extraction. Also, intact colonies of *Trididemnum solidum* and *Lissoclinum* sp. were also extracted with EtOH and then total extracts analysed.

Isolation of amino acids and GC/MS analysis. Acidified (dil HOAc) extracts were concd to remove EtOH and aq. residues placed on columns of Dowex 50-X8 (H^+ form, washed with H_2O and the total amino acids eluted with NH_4OH (2 M). This eluate was dried, resuspended in H_2O and applied to a column of Dowex 1-X8 (OAc^- form) which was washed with H_2O and subsequently eluted with HOAc (2 M) to yield a fraction were prepared using a mixture of MeCN–BSTFA–TMCS (100:100:1) and heating at 70° for 15 min. Other derivatives were prepared using standard methods [10]. Aliquots were subjected to GC/MS analysis using packed columns (2 m × 2 mm i.d.) of 3% Dexsil (300) on Gas Chrom Q (80–100) or 2% OV-17 on Gas Chrom Q (80–100). He flow was 30 ml/min and the oven programmed from 100° to 250° at 10°/min with injector, line and jet separator all at 250°. MS were recorded at 70 eV, source 220° scanned at 6 sec/decade. The relative abundancies of 3-(*N*-methylamino)glutarate in various algal extracts given in Table 1 were estimated from GC/MS total ion currents in relation to the peak height of TMSi glutamate. The TMSi derivative of the major compound eluting after TMSi glutamate showed the following spectrum: *m/z* 377 (0.1% RI, M^+), 362 (15, $M^+ - Me$), 347 (0.1), 331 (0.5), 320 (4), 305 (1), 302 (3), 272 (3), 246 (100, $M^+ - CH_2COOTMSi$), 230 (12), 202 (12), 200 (3), 192 (12), 188 (12), 174 (20), 170 (10), 147 (52). The spectrum of the diMe ester showed ions at *m/z* 189 (3% R.I., M^+), 174 (10, $M^+ - Me$) and 113 (100, $M^+ - CH_2COOMe$). The spectrum of the TAB derivative showed ions at *m/z* 369 (18, M^+), 295 (22), 272 (57), 267 (55), 254 (35), 240 (42), 221 (20), 198 (22), 180 (25), 154 (40), and 117 (100) and the molecular ion mass measured (using a high resolution instrument) at 369.1763 (calc. for $C_{16}H_{26}NO_5F_3$ 369.1761) corresponding to addition of two butyl ester and one trifluoroacetyl groups to the original molecule $C_6H_{11}NO_4$.

Synthesis of 3-(N-*methylamino*)*glutaric acid* (**1**). Diethylglutaconate (0.5 g), $MeNH_2$ (8 g) and EtOH (17 ml) were stirred at 25° for 24 hr. The mixture was evapd to an oil which was refluxed in HCl (25 ml, 5 M) for 1 hr, extracted with Et_2O and evapd to dryness. The residue was dissolved in H_2O (20 ml) and allowed to percolate through a column of Dowex 50-X 8 (H^+ form), washed with H_2O and eluted with NH_4OH (2 M). The product was further purified by adsorption on and elution from Dowex 1 (OAc^- form) and recrystallization from aq. EtOH to give colourless prisms (160 mg), mp 177–179°. ^1H NMR (D_2O): δ 3.2 (7 H, *m*, N–Me + 2 × $-CH_2-$), 4.26 (1 H, *t*, J = 6 Hz, CH). The TAB derivative had a M^+ which was measured at 369.1761 (calc. for $C_{16}H_{26}NO_5F_3$: 369.1761) and a fragmentation pattern and GC R_t identical to that of the natural product. The diMe ester and TMSi derivatives also had GC R_t and MS indistinguishable from those of the natural product. The synthetic and natural products co-chromatographed on Si gel and cellulose TLC plates developed in BuOH–HOAc–H_2O (12:3:5, R_f 1.7 and 2.3, respectively). Spots were visualized with I_2 vapour and gave only a weak yellow colour with ninhydrin.

Acknowledgements—This work was made possible by the laboratory and logistic support of the Australian Institute of Marine Science and the Lizard Island Research Station, and the assistance of these organizations is warmly acknowledged. I also thank Professors R. A. Lewin, Scripps Institution of Oceanography, La Jolla, California and D. J. Griffiths, James Cook University, Townsville, Queensland for providing plant material for analysis; Dr. Patricia Mather for identification of *D. molle*; and Prue Kell and Lynne Cambridge for technical assistance. This work was carried out with support from an Australian Marine Sciences and Technologies (AMSTAC) Grant.

REFERENCES

1. Newcomb, E. H. and Pugh, T. D. (1975) *Nature* **253**, 533.
2. Lewin, R. A. and Withers, N. W. (1975) *Nature* **256**, 735.
3. Thorne, S. W., Newcomb, E. H. and Osmond, C. B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 575.

4. Withers, N., Vidaver, W. and Lewin, R. A. (1978) *Phycologia* **17**, 167.
5. Lewin, R. A. (1976) *Nature* **261**, 697.
6. Antia, N. J. (1977) *Br. Phycol. J.* **12**, 271.
7. Perry, G. J., Gillan, F. T. and Johns, R. B. (1978) *Br. Phycol. J.* **14**, 369.
8. Kuehn, G. and Hoyer, E. (1967) *Makromol. Chem.* **108**, 84.
9. Takemoto, T. and Sai, T. (1965) *J. Pharm. Soc. (Jpn)* **85**, 33.
10. Darbre, A. (1978) in *Handbook of Derivatives for Chromatography* (Blau, K. and King, G. S., eds.) pp. 65–66. Heyden, London.

Phytochemistry, Vol. 20, No. 5, pp. 1127–1128, 1981.
Printed in Great Britain.

0031-9422/81/051127-02 $02.00/0
Pergamon Press Ltd.

GLUCOSE-1-BENZOATE AND PRUNASIN FROM *PRUNUS SEROTINA*

STEPHEN B. HORSLEY* and JERROLD MEINWALD†

* USDA, Forest Service, Northeastern Forest Experiment Station, Warren, PA 16365, U.S.A.;
† Department of Chemistry, Cornell University, Ithaca, NY 14853, U.S.A.

(*Revised received* 29 *June* 1980)

Key Word Index—*Prunus serotina*; Rosaceae; Black cherry; benzoate ester; cyanogenic glycoside; prunasin; allelopathy.

Abstract—β-D-Glucopyranose 1-benzoate and prunasin have been isolated from the leaves of *Prunus serotina*. Both can yield benzoic acid, a potential allelopathic inhibitor of *Acer rubrum*.

Benzoic acid has been known as a plant growth inhibitor since at least 1914 when Shorey [1] and later Walters [2] isolated it from soils 'fatigued' by citrus. It is known to occur in plants in esterified form [3], and is produced as the oxidation product of benzaldehyde, released on enzymatic hydrolysis of the mandelonitrile glycosides [4, 5]. Benzoic acid has subsequently been shown to inhibit growth of peach seedlings (*Prunus* spp.) [6] and of mycorrhizal fungi [7], as well as the process of ion uptake in isolated barley (*Hordeum vulgare* L.) root tips [8]. Recently, while investigating the allelopathic effects of black cherry (*Prunus serotina* Ehrh.) on red maple (*Acer rubrum* L.), one of us (S.B.H.) found that benzoic acid, released during natural senescence of black cherry leaves, could inhibit the growth of red maple seedlings in concentrations as small as 0.1 mM [9]. We were interested to know the potential sources of benzoic acid in black cherry leaves.

In the present investigation 600 g fr. wt of leaves yielded 22.45 g of crude defatted extract, from which we have isolated two compounds from which benzoic acid could be produced during senescence: β-D-glucopyranose 1-benzoate (**1**) (488 mg) and prunasin (**2**) (337 mg). The former has been reported previously as a synthetic product [10–12], as a secretion of certain insects (*Periplaneta americana* L., *Blatta orientalis* L.) [13], and more recently from the needles of the gymnosperm, *Pinus contorta* Dougl. [14]. However, to our knowledge it has never previously been found in an angiosperm. Prunasin (**2**) was reported 70 years ago [15, 16] to be a constituent of black cherry bark and leaves. We confirm this early report, and provide MS and ^{13}C NMR spectral data to substantiate the structural assignment. Fully assigned ^{13}C NMR spectra of any cyanogenic glycoside have only recently been reported [17]. Both **1** and **2** were isolated by repeated chromatography of an aqueous ethanol extract of black cherry leaves. The IR spectrum of **1** indicated the presence of a monosubstituted aromatic ring, carbonyl, and hydroxyl functions. Although no molecular ion was present in the MS of **1**, prominent ions at *m/e* 105 and 122, corresponding to $C_7H_5O^+$ and $C_7H_6O_2^+$ fragments, respectively, suggested the presence of a benzoate ester. Signals in the ^1H NMR spectrum [δ8.09 (2H, *dd*, $J = 2$ Hz, $J = 8$ Hz) and 7.64 (3H, *m*)] and in the ^{13}C NMR (δ165.09, 134.14, 129.89, 129.48, 129.11) confirmed this part structure. The six remaining signals in the ^{13}C NMR (δ95.35, 78.19, 76.69, 72.88, 69.87, 60.96) suggested the presence of a hexose sugar, but a large HOD peak in the ^1H NMR spectrum prevented a complete analysis of the corresponding proton resonances. Acetylation yielded a chloroform-soluble tetraacetate, **3**, whose ^1H NMR spectrum revealed five methine and two methylene protons between δ3.9 and 5.8, in addition to the signals for the acetyl methyls and benzoate ester. Identification was confirmed by comparison of the physical properties of **1** and **3** with those reported in the literature (mp [13, 18], OR [11], IR [13, 18], ^1H NMR [14]).

Enzymatic hydrolysis of **2** in the presence of picrate paper suggested that it was a cyanogenic glycoside. Although a molecular ion was not observed in the mass