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LUNULARIC ACID DECARBOXYLASE FROM THE LIVERWORT *CONOCEPHALUM CONICUM*

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Key Word Index—*Conocephalum conicum*; liverwort; lunularic acid decarboxylase; lunularin; pinosylvic acid; inhibitor.

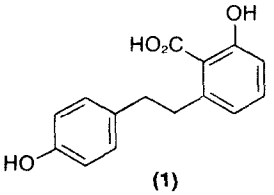
Abstract—Some properties of a preparation of an enzyme, lunularic acid decarboxylase, from the liverwort *Conocephalum conicum* are described. The enzyme is normally bound and could be solubilized with Triton X-100; at least some of the bound decarboxylase activity appears to be associated with chloroplasts. For lunularic acid the enzyme has K_m $8{\cdot}7 \times 10^{-5}$ M (pH 7·8 and 30°). Some substrate analogues have been tested but no other substrate was found. Pinosylvic acid is a competitive inhibitor for the enzyme, K_i $1{\cdot}2 \times 10^{-4}$ M (pH 7·8 and 30°). No product inhibition was observed. Lunularic acid decarboxylase activity has also been observed with a cell-free system from *Lunularia cruciata*.

INTRODUCTION

THE DIHYDROSTILBENE carboxylic acid, lunularic acid (**1**), is a natural growth inhibitor and dormancy factor of liverworts.[1,2] Liverworts contain no detectable abscisic acid (**2**)[3] which has a similar function in higher plants.[1] Investigations of the biosynthesis[4] and further metabolism[5] of lunularic acid in the thallose liverwort *Lunularia cruciata* have shown that the observed variation in the amount of extractable lunularic acid with photoperiod[6] is probably due to regulation of the biosynthetic pathway. Further metabolism of administered lunularic acid proceeded at very similar rates and to the same products in both light and dark.[5] These time-course studies of the catabolism of lunularic acid in thalli segments of *L. cruciata* showed that decarboxylation to the dihydrostilbene, lunularin (**3**), was the first step.[5] We have now been able to prepare a solubilized crude preparation of the previously unknown enzyme responsible for this decarboxylation, lunularic acid decarboxylase (LNA-decarboxylase), from the thallose liverwort *Conocephalum conicum* and report here some of its properties. To the authors' knowledge this is the first investigation of an aromatic decarboxylase from green plants. Aromatic decarboxylases of microorganisms and animals have been investigated.[7] In particular, orsellinic acid (**4**) decarboxylase from the fungus *Gliocladium roseum*[7] and the lichen *Umbilicaria pustulata*,[8]

[1] PRYCE, R. J. (1972) *Phytochemistry* **11**, 1759 and references therein.
[2] VALIO, I. F. M., BURDEN, R. S. and SCHWABE, W. W. (1969) *Nature* **223**, 1176.
[3] RYBACK, G. (1972) *J. C. S. Chem. Comm.* 1190 for revised stereochemistry of abscisic acid.
[4] PRYCE, R. J. (1971) *Phytochemistry* **10**, 2679.
[5] PRYCE, R. J. (1972) *Phytochemistry* **11**, 1355.
[6] VALIO, I. F. M. and SCHWABE, W. W. (1970) *J. Exp. Botany* **21**, 138.
[7] PETTERSSON, G. (1965) *Acta Chem. Scand.* **19**, 2013 and references therein.
[8] MOSBACH, K. and EHRENSVÄRD, U. (1966) *Biochem. Biophys. Res. Commun.* **22**, 145.

and 6-methylsalicylic acid (**5**) decarboxylase from *Penicillium patulum*[9] involve substrates of related structure to lunularic acid.



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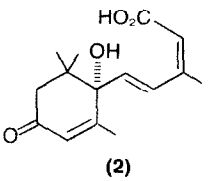

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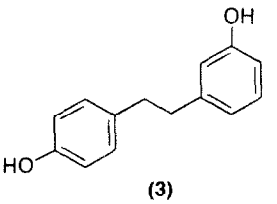

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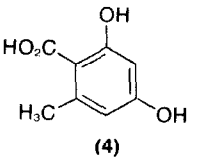

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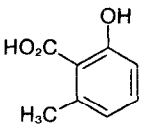

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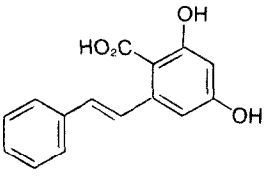

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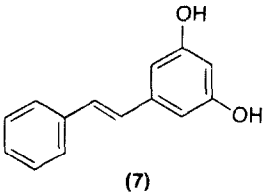

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RESULTS AND DISCUSSION

Initially, LNA-decarboxylase activity was detected in the 1000 *g* supernatant fraction from a cell-free system of *L. cruciata* thalli. The LNA-decarboxylase activity of this preparation required none of the added co-factors, ATP, NADPH, NADH or Mg^{2+}. Similarly, 6-methylsalicylic acid and orsellinic acid decarboxylases have no co-factor requirements.[7–9] No metabolites other than the decarboxylation product, lunularin (**3**), could be observed with the *L. cruciata* system using lunularic acid-[^{14}C] as substrate. The common thallose liverwort *Conocephalum conicum* was chosen as a source of LNA-decarboxylase for the more detailed investigation which follows. Lunularic acid had previously[1] been shown to occur in thalli of *C. conicum* and now the predicted[5] co-occurrence of lunularin (**3**) has been confirmed by GC–MS analysis of extracts. It is probable that lunularin[5] and LNA-decarboxylase are of widespread occurrence in liverworts, as seems to be the case with lunularic acid itself.[1] Lunularin has also been shown to be present in *L. cruciata*[5] and *Marchantia polymorpha*.[10]

LNA-decarboxylase activity in cell-free homogenates of *C. conicum* is completely precipitated at 105000 *g*, therefore it is not a "soluble" enzyme. Solubilization of the enzyme activity could be efficiently (*ca* 75%) effected by Triton X-100 treatment of the 105000 *g* pellet. This solubilized preparation of LNA-decarboxylase is the preparation whose properties are described below. A typical preparation had a specific activity of $1{\cdot}6 \times 10^{-2}$ U/mg (at pH 7·8 and 30°); this and other results were determined by a GLC assay and lunularin was the only detected product (GC–MS). For reasons of substrate solubility and the amounts of substrate required for the GLC assay, assays of the solubilized LNA-decarboxylase were conducted at pH 7·8. However, practically no difference in activity of the preparation was observed at pH 6·0 when a small amount of acetone was used as co-solvent for the substrate. The stoichiometry of the LNA-decarboxylase reaction (**1** = **3** + CO_2) was determined by a combination of Warburg manometry to measure carbon diox-

[9] VOGEL, G. (1971) Doctoral Thesis, University of München.
[10] HOPKINS, B. J. and PEROLD, G. W. (1974) *J. C. S. Perkin* **1**, 32.

ide evolution and GLC analysis to measure disappearance of lunularic acid and appearance of lunularin. Good Michaelis–Menten kinetics were shown by the solubilized LNA-decarboxylase preparation which had K_m for lunularic acid $8{\cdot}7 \times 10^{-5}$ M (at pH 7·8 and 30°).

The structurally related aromatic carboxylic acid, 6-methyl-salicylic acid (**5**) was not decarboxylated by the LNA-decarboxylase preparation and neither was the even more closely related stilbene carboxylic acid, pinosylvic acid (**6**). However, pinosylvic acid is a good, competitive inhibitor of LNA-decarboxylase, K_i $1{\cdot}2 \times 10^{-4}$ M (at pH 7·8 and 30°). Although pinosylvic acid[11] is as yet unknown as a natural product it has been proposed as a biosynthetic precursor of the natural plant stilbene pinosylvin (**7**), its decarboxylation product.[12,13] 6-Methylsalicylic acid does not inhibit the decarboxylation of lunularic acid by the decarboxylase, neither does abscisic acid (**2**) whose structural similarities with lunularic acid have been noted elsewhere;[1] abscisic acid was apparently unaffected by the LNA-decarboxylase. Therefore, the inhibition of liverwort growth caused by abscisic acid is probably not due to blocking of LNA catabolism by inhibition of the decarboxylase. No inhibition of LNA-decarboxylase was observed with the decarboxylation product, lunularin (**3**).

The location of the apparently bound LNA-decarboxylase within the liverwort cell remains uncertain. However, an experiment did indicate that at least some of the bound activity was associated with chloroplasts.

EXPERIMENTAL

Lunularic acid for this work was prepared by hydrogenolysis[5] of hydrangenol, it recrystallized from EtOH/H_2O with no solvent of crystallization m.p. 194–200° (decomp.) (Found: C, 70·2; H, 5·8. Calc. for $C_{15}H_{14}O_4$: C, 69·8; H, 5·5%). The sample of pinosylvic acid was recrystallized before use.[11] *Conocephalum conicum* was cultivated on compost in humidity boxes at 23–25° under 12 hr light/day (450 lx from G.E.C. daylight fluorescent lights). Buffers used were 0·05 M KH_2PO_4/K_2HPO_4 pH 5·5 (pH 5·5 phosphate buffer) and 0·05 M Tris–HCl pH 7·8 (pH 7·8 Tris buffer). The preparation and all manipulations of the enzyme solutions were carried out at 4°. Protein determinations were carried out by the method of Lowry[14] using bovine serum albumin as standard.

Lunularin (**3**) *in* C. conicum. Fresh, washed thalli of *C. conicum* (470 g) were extracted as before[5] to give a "weak acid" fraction (94 mg), an aliquot of which was methylated and analyzed for lunularin by GLC[5] and GC–MS[15] as previously described. The presence of lunularin was confirmed.

LNA-decarboxylase from C. conicum; *solubilized LNA-decarboxylase.* Fresh thalli of *C. conicum* (5·8 g) were homogenized in a Potter homogenizer in pH 7·8 Tris buffer (5 ml). The homogenate was filtered through a double layer of cheese cloth and the filtrate and washings (buffer 2 × 2·5 ml) were collected. The total filtrate was centrifuged at 100 *g* for 10 min and the pellet was discarded; the supernatant was then centrifuged at 105000 *g* for 1 hr to give an active pellet and inactive supernatant. After resuspending the 105000 *g* pellet in pH 7·8 Tris buffer containing 0·3% Triton X-100 (10 ml) it was stirred for 18 hr, then centrifuged at 105000 *g* for 1 hr. The final supernatant which contained *ca* 75% of the LNA-decarboxylase activity (the pellet contained the remainder) was then passed through a column (51 × 2 cm) of Sephadex G25 (medium), made up in pH 7·8 Tris buffer, to remove Triton X-100. Elution of the column was with pH 7·8 Tris buffer. The green fractions coming off the column in its void volume contained all the decarboxylase activity and these were combined and brought to 90% saturation with $(NH_4)_2SO_4$. The protein that precipitated was collected by centrifugation at 15000 *g* for 10 min, then it was redissolved in pH 7·8 Tris buffer (5 ml) to give the soluble LNA-decarboxylase preparation of the type used below. This present preparation contained 1 mg protein/ml and had a specific activity of $1{\cdot}59 \times 10^{-2}$ U/mg under standard assay conditions. A preparation of this soluble LNA-decarboxylase has been stored in the dark at 4° for up to 2 months with no detectable loss of activity.

[11] Harris, T. M. and Carney, R. L. (1967) *J. Am. Chem. Soc.* **89,** 6734.
[12] Birch, A. J. and Donovan, F. W. (1953) *Australian J. Chem.* **6,** 360.
[13] Hillis, W. E. and Yazaki, Y. (1971) *Phytochemistry* **10,** 1051.
[14] Lowry, O. H., Rosebough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[15] Pryce, R. J. (1973) *Phytochemistry* **12,** 507.

LNA-decarboxylase from chloroplasts. Fresh thalli of *C. conicum* (6·8 g) were homogenized as above in pH 7·8 Tris buffer containing 0·4 M sucrose and the cheese cloth filtrate was collected as above. This filtrate was centrifuged at 100 *g* for 10 min and the pellet was discarded. In this account the decarboxylase activity, determined under standard assay conditions, of the subsequent fractions referred to is quoted in parenthesis (as μmoles LNA reacted or μmoles lunularin formed). The 100 *g* supernatant (10·8 ml) was centrifuged at 2000 *g* for 10 min to give a pellet which was resuspended in the Tris–sucrose buffer above (10·8 ml), and a supernatant (10·8 ml) (0·036 μmol). The resuspended 2000 *g* pellet was washed 2 × in Tris–sucrose buffer (10·8 ml) by two sequences of centrifugation at 2000 *g* for 10 min and resuspension of the pellet in the Tris–sucrose buffer (10·8 ml) except that after the last wash the pellet was resuspended in pH 7·8 Tris buffer (10·8 ml) alone. The activities of the washings were: first wash (0·013 μmol), second (0·003 μmol). An aliquot (1 ml) of the washed resuspended "chloroplast pellet" was taken for electron microscopy and standard enzyme assay. To the remaining washed resuspended "chloroplast pellet" (9·8 ml) (0·009 μmol) was added Triton X-100 (2 drops to make *ca* 0·3% soln) and it was then stirred for 18 hr before centrifuging it at 105000 *g* for 1 hr. The 105000 *g* pellet thus obtained was discarded, the 105000 *g* supernatant was passed down a column of Sephadex G-25 as above, and the protein coming off in the void volume was collected by $(NH_4)_2SO_4$ precipitation as above to give a solubilized preparation from the washed chloroplast pellet in pH 7·8 Tris buffer (9·8 ml) (0·006 μmol, 67% of the activity of the washed resuspended chloroplast pellet above). Electron microscopy of the washed chloroplast pellet showed minor contamination with what appeared to be cell wall fragments.

Assay of LNA-decarboxylase. All incubations were conducted at 30°. Substrate and enzyme solns pH 7·8 Tris buffer were equilibrated for 10 min at 30° before mixing. Reactions were stopped by addition of 1 N HCl (1 ml) then extracted with EtOAc (3 × 1·5 ml) and the EtOAc extract dried (Na_2SO_4) before evaporating it to dryness *in vacuo* at 30°. In the case of the 6-methylsalicylic acid incubation below, Et_2O was used for the extraction to avoid evaporating any *m*-cresol decarboxylation product. The extracts in MeOH were methylated for 48 hr at room temp. with excess CH_2N_2/Et_2O to ensure complete methylation, they were then dissolved in MeOH (200 μl) for GLC analysis. Control incubations of the enzyme preparation above and the substrate or inhibitors alone were always carried out. No substance similar in GLC retention time to substrates, products or inhibitors was noted with the enzyme alone and, unless otherwise noted, no change in substrates or inhibitors was noted (GLC analysis) in the absence of the enzyme. GLC of methylated extracts was carried out with a Varian aerograph 1400 instrument fitted with an FID detector and a silanized glass column (1·5 × 3 mm i.d.) packed with 1% OV 17 adsorbed onto Gas Chrom Q (100–120 mesh). The column oven temp. was 200° and injector and detector heaters were kept at 250°, N_2 flow rate was 45 ml/min. Under these conditions the R_fs of the fully methylated derivatives of the compounds described were as follows: lunularic acid (**1**, 11·0 min), lunularin (**3**, 2·75 min), pinosylvic acid (**6**, 30·9 min), pinosylvin (**7**, 6·4 min), abscisic acid (**2**, 3·6 min), 6-methylsalicylic acid (**5**, 11·6 min at 100°), *m*-cresol (0·7 min at 100°). Quantitative GLC analysis was by peak area measurements. The relative FID detector response for fully methylated lunularic acid and lunularin was calibrated using a series of methylated mixtures containing different known amounts of the two compounds and it was found that: mole fraction of lunularic acid decarboxylated or mole fraction of lunularin formed = 0·96 (lunularin peak area)/(lunularin + lunularic acid peak areas). GC–MS of enzyme incubation extracts confirmed lunularin as the sole observed product from lunularic acid.

One enzyme unit, *U*, is defined as the amount of protein that catalyses the decarboxylation of 1 μmol of lunularic acid per min under the standard assay conditions.

The *standard assay* referred to above was the incubation for 75 min at 30° of the enzyme solution (0·1 ml), the substrate (lunularic acid) soln (*ca* 1·5 mM, 0·2 ml) both in pH 7·8 Tris buffer, and pH 7·8 Tris buffer (0·2 ml). Work up and GLC assay were always as described above. Under these standard conditions but varying the amount of the soluble LNA-decarboxylase solution ($1·59 \times 10^{-2}$ U/mg protein, 1·00 mg protein/ml) from 0 to 0·2 ml a linear plot of μmoles lunularic acid decarboxylated vs ml of enzyme soln was obtained up to 0·15 ml of the enzyme soln where 0·18 μmol of lunularic acid had reacted (initially 0·31 μmol of lunularic acid put into the assay). In an assay consisting of the enzyme soln just described (0·4 ml), pH 7·8 Tris buffer (0·8 ml) and lunularic acid [1·24 μmoles in pH 7·8 Tris buffer (0·8 ml)] incubation for different times gave a good linear plot of μmoles of lunularic acid reacted vs time for up to 80 min reaction when 0·51 μmol or 43% of the lunularic acid had been decarboxylated. With the same enzyme solution above (0·4 ml), pH 7·8 Tris buffer (1·5 ml), and lunularic acid [0·16 μmol in pH 7·8 Tris buffer (0·1 ml)] the plot of lunularic acid reacted vs time was linear only up to 10 min of incubation when 0·047 μmol or 32% of the lunularic acid had been decarboxylated. Unless otherwise stated, in all other assays the total volume of the assay was kept at 0·5 ml by adjusting the volumes of substrate, enzyme and buffer solns.

Assay at pH 6·0. A standard assay (above) at pH 7·8 using a soluble LNA-decarboxylase preparation ($1·31 \times 10^{-2}$ U/mg, 1·25 mg protein/ml) with 0·31 μmol of lunularic acid in the assay decarboxylated 0·12 μmoles of lunularic acid. In a similar assay with the same enzyme solution (in pH 7·8 Tris buffer) but with lunularic acid in pH 5·5 phosphate buffer containing 4% acetone, and pH 5·5 buffer containing 4% acetone (the incubation mixture was pH 6·0) 0·13 μmol of lunularic acid was decarboxylated.

Stoichiometry of the decarboxylase. The soluble LNA-decarboxylase in pH 7·8 Tris buffer (0·2 ml, $1·24 \times 10^{-2}$ U/mg, 1·55 mg protein/ml) and lunularic acid (0·8 ml, 1·19 μmol in pH 5·5 phosphate buffer containing 4% acetone) were incubated for 75 min at 30° and worked up for GLC analysis as usual. In a parallel experiment the

same assay mixture was subjected to Warburg manometry (enzyme initially in the side-arm of reaction flask) to measure CO_2 evolution in 75 min at 30°. GLC analysis gave 0·28 μmol of lunularic acid decarboxylated and 0·28 μmol of lunularin produced during the incubation and Warburg manometry gave 0·25 μmol CO_2 evolved for the parallel incubation. The working pH of both incubations was pH 6·0.

K_m *determination.* Six incubations were carried out at 30° for 10 min each consisting of LNA-decarboxylase (0·1 ml, $1·59 \times 10^{-2}$ U/mg, 1·00 mg protein/ml in pH 7·8 Tris buffer), six different lunularic acid solutions pH 7·8 Tris buffer (0·05–0·40 ml, 0·078–0·62 μmol) and pH 7·8 Tris buffer to 0·5 ml total incubation volume. Lineweaver–Burk plots of three such experiments gave a mean value for K_m $8·7 \times 10^{-5}$ M and V_{max} 1·8 nmol/min. The maximum amount of reaction observed was 14·6% under these conditions.

Substrate analogue and inhibitor studies. 6-Methylsalicylic acid (**5**) and pinosylvic acid (**6**) were incubated under the standard assay conditions above with LNA-decarboxylase (0·2 ml, $1·59 \times 10^{-2}$ U/mg, 1·00 mg/ml). No reaction occurred with 6-methylsalicylic acid and no decarboxylation of pinosylvic acid to pinosylvin (**7**) was observed above that in the control (1% decarboxylation of **6**); longer incubation (75 min) with pinosylvic acid gave a similar result. In a similar experiment to the K_m determination above and another parallel experiment in which to each incubation was added pinosylvic acid in pH 7·8 Tris buffer (0·1 ml, 0·15 μmol) and LNA-decarboxylase (0·1 ml, $1·31 \times 10^{-2}$ U/mg, 1·25 mg protein/ml) a Lineweaver–Burk plot showed that pinosylvic acid was a competitive inhibitor, K_i $1·2 \times 10^{-4}$ M. The same K_i was obtained in a confirmatory experiment when 0·075 μmol of pinosylvic acid was added to each incubation. Similar experiments to this K_i determination but with abscisic acid (**2**) (0·15 μmol) and 6-methylsalicylic acid (**5**) (0·19 μmol) showed no inhibition of LNA-decarboxylase; abscisic acid was apparently (GLC) unaffected by the decarboxylase. No inhibition of LNA-decarboxylase, in a standard assay, could be detected in the presence of lunularin (0·43 μmol). Work-up in this latter case was accompanied by the addition of a known amount of *n*-octacosane as an internal GLC standard to facilitate measurement of the change in the amount of lunularin in the presence of the excess of lunularin added to the assay.

LNA-decarboxylase activity from L. cruciata. Fresh thalli tips (4 g) of short-day *L. cruciata*[4] and sand (8 g) were ground together in a mortar with pH 7·8 Tris buffer (4 ml) then filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 1000 *g* for 5 min. Three incubations were carried out for 1 hr each at 30° with the 1000 *g* supernatant (0·35 ml each) and lunularic acid-[^{14}C] ($1·52 \times 10^4$ dpm each, $2·32 \times 10^6$ dpm/μmol, labelled biosynthetically from L-phenylalanine-[U-^{14}C][4,5]) added dry to each incubation tube. The three incubations, which were all of final volume 0·5 ml in pH 7·8 Tris buffer, contained the following additions: incubation (1) 1 mM in each of the cofactors ATP, NADPH, NADH, $MgCl_2$ and sucrose, and 3 mM in 2-mercaptoethanol; (2) the cofactors above only; (3) 2-mercaptoethanol above only. A fourth incubation [(4)] was as (1) above except that the 1000 *g* supernatant had been kept in a boiling water bath for 10 min prior to the incubation. Each incubation was stopped and worked up as for the *C. cephalum* assay above to give an EtOAc extract which was subjected to SiO_2 TLC-radioautography as previously described[4,5] using EtOAc–$CHCl_3$–HOAc (15:5:1) as developing solvent. The radioautograms of each of the incubations (1)–(3) showed only two peaks which corresponded to lunularic acid and lunularin (also seen by the UV fluorescence) and the percentage of lunularin formed was estimated from the areas of the radioautogram peaks: (1) 15%, (2) 13%, (3) 13%. The incubation (4) with boiled enzyme showed no conversion to lunularin. The band corresponding to lunularin in the above TLC of incubation (1) was extracted[4] and rechromatographed using the developing solvent diisopropyl ether–HOAc (19:1) and radioautography showed only one radioactive component coincident with the lunularin peak (UV fluorescence detection). This latter band corresponding to lunularin-[^{14}C] was extracted and rechromatographed using the developing solvent Bz–MeOH–HOAc (20:4:1) and again radioactivity was coincident with the lunularin band.

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