Phytochemistry, 1970, Vol. 9, pp. 2477 to 2481. Pergamon Press. Printed in England.

PULVINAMIDE AND POSSIBLE BIOSYNTHETIC RELATIONSHIPS WITH PULVINIC ACID*

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(*Received* 30 *January* 1970, *in revised form* 19 *March* 1970)

Abstract—A new pulvinic acid derivative, pulvinamide, has been identified as a constituent of *Pseudocyphellaria crocata*. With the possibility that pulvinamide is a key biosynthetic intermediate a more detailed pathway for the biogenesis of pulvinic acid and its derivatives is proposed, i.e. stepwise aldol-type of condensation of 1 mole phenylpyruvic acid with 1 mole pyridoxal-bound phenylalanine to give a quinoneimine analogue of polyporic acid which, after oxidative cleavage, would yield bound pulvinamide as the immediate precursor of the free pulvinic acid derivatives.

INTRODUCTION

DURING the isolation of various orsellinic acid esters from the lichen *Pseudocyphellaria crocata*[1] considerable amounts of calycin and traces of pulvinic acid and as yet unidentified yellow pigments were found to accompany the esters in the sodium carbonate and sodium hydroxide fractions of ether extracts. One of these, designated compound 21, has now been isolated and identified as pulvinamide (pulvinic acid amide).

In view of our interest in the biosynthesis of pulvinic acid derivatives[2,3] the possible origin off the amide group will be discussed in relation to the biosynthetic mechanism of polyporic acid and pulvinic acid formation.

RESULTS

Pulvinamide (m.p. 220–221° dec) was purified via TLC of the mother liquor of calycin fractions which had been eluted from a column of non-activated silicic acid with methylene chloride.[1]

A mass spectrum of the compound showed a molecular ion *m/e* 307·0841 ($C_{18}H_{13}NO_4$ 307·0845) which further fragmented to the ion *m/e* 290·0572 ($C_{18}H_{10}O_4$ 290·0579). The fragmentation pattern below *m/e* 290 resembled that of pulvinic dilactone ($m^+ = 290{\cdot}0579$) reported by Letcher and Eggers.[4] When a mass spectrum of pulvinic dilactone was run under comparable conditions, the similarity of the spectra became very striking indeed (Fig. 1). These observations strongly suggested the electron-impact formation of a pulvinic dilactone ion (*m/e* 290) by elimination of NH_3 (corresponding to 17 mass units). Two alternative structures could possibly account for such a mechanism, namely either the amide of pulvinic

* Lichen Substances III, issued as N.R.C.C. No. 11473. Paper II of this series appeared in *Can. J. Botany* **45**, 59 (1967).

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[2] W. S. G. MAASS, G. H. N. TOWERS and A. C. NEISH, *Ber. Dtsch. Bot. Ges.* **77**, 157 (1964).
[3] W. S. G. MAASS and A. C. NEISH, *Can. J. Botany* **45**, 59 (1967).
[4] R. M. LETCHER and S. H. EGGERS, *Tetrahedron Letters* 3541 (1967).

acid or the less likely structure of a pulvinic dicarboxylic acid imide. The first alternative is favoured by the formation of a monomethyl ether (m.p. 218° dec) upon treatment with diazomethane. The NMR spectra of both pulvinamide and the *O*-methyl derivative show the presence of 10 aromatic protons, and two further exchangeable hydrogens appear as broad signals at 2·67 and 2·90 τ and 2·60 and 3·15 τ respectively and are typical of an amide group. The i.r. spectrum of pulvinamide shows both O—H and N—H signals at 3466, 3447, 3360 and 3328 cm^{-1} respectively. The *O*-methyl derivative, which contains only the NH_2 group, also shows similar peaks (at 3411, 3302, 3253 and 3194 cm^{-1}). The amide carbonyl frequency in pulvinamide is in an area (1670 cm^{-1}) where most pulvinic acid derivatives, such as vulpinic

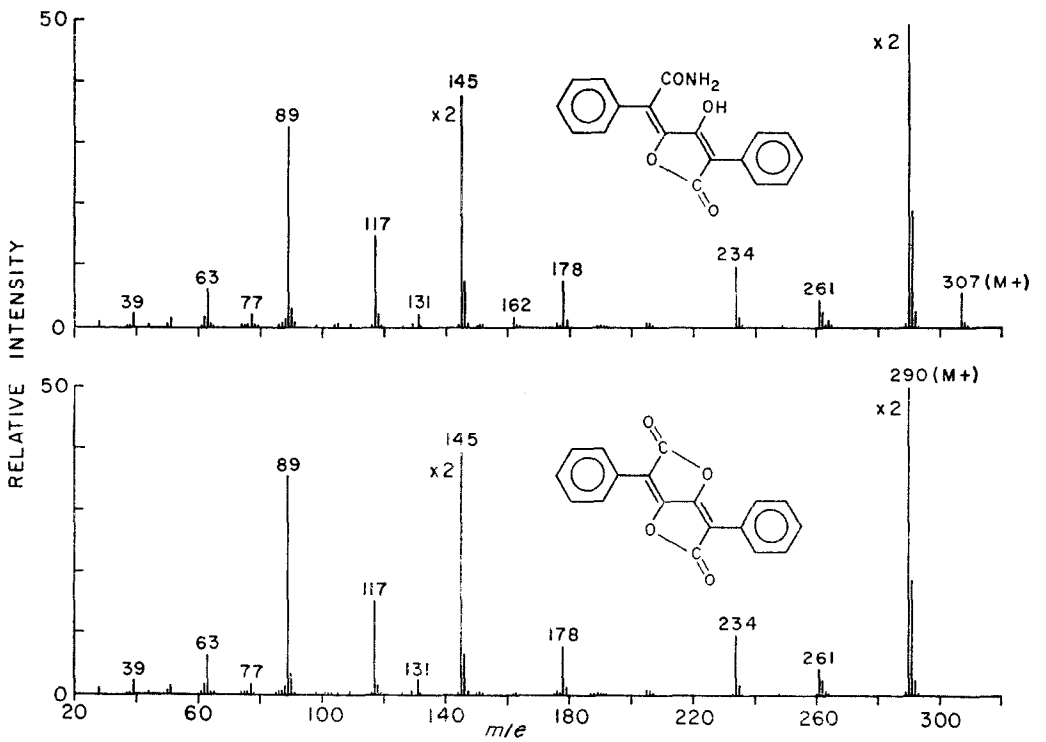

FIG. 1.

acid,[5] show a hydrogen-bonded carbonyl group that disappears in the *O*-methyl derivatives. *O*-Methylpulvinamide displays a strong absorption at 1659 cm^{-1} consistent with that of an amide. The identity of the natural compound was finally proved by synthesis of pulvinamide from pulvinic dilactone and ammonia, giving identical melting points, u.v., i.r., NMR and mass spectra as well as furnishing an *O*-methyl derivative identical with that obtained from the natural sample.

Pulvinamide can be sublimed; however, some decomposition into pulvinic dilactone, pulvinic acid and unidentified yellow compounds occurs. Pulvinamide was smoothly converted into pulvinic dilactone on treatment with hot acetic anhydride.

[5] B. ÅKERMARK, *Acta Chem. Scand.* **15**, 1695 (1961).

DISCUSSION

Besides rhizocarpic acid (*N*-pulvinyl-L-phenylalanine methyl ester) and epanorin (*N*-pulvinyl-L-leucine methyl ester), pulvinamide is the third natural pulvinic acid derivative to contain nitrogen. It appears to be a minor constituent in numerous species of the genus *Pseudocyphellaria*, including species with green algae such as *Pseudocyphellaria aurata*.[1] Epanorin has only been found in *Lecanora epanora*, whereas rhizocarpic acid is fairly widely distributed among lichens,[6] perhaps owing to the biosynthetic relationship of the phenylalanine residue with the pulvinyl group.

The question of the biosynthetic origin of the nitrogen in pulvinamide is particularly intriguing since it could be introduced at a stage after *or* prior to pulvinic acid formation. In the former case pulvinic acid would appear as the immediate precursor of pulvinamide while in the latter case pulvinamide would be formed parallel, if not prior to, pulvinic acid. This would require a direct involvement of phenylalanine in the formation of intermediates which is not in serious contradiction to the published data on pulvinic acid biosynthesis.[2,3,7] Moreover, in a study on the biosynthesis of calycin,[1] it was found that vulpinic-^{14}C acid (as well as pulvinic-^{14}C acid) gave rise to radioactive calycin (containing up to 23% of the ^{14}C taken up) only after a lag phase during which no activity could be detected in either calycin, pulvinic dilactone or pulvinic acid extracted from the lichen by organic solvents. This result suggested the formation of bound pulvinic acid prior to its conversion into calycin. The following modified pathway of pulvinic acid biosynthesis takes into account such bound forms of pulvinic acid and its precursors.

Alternative to the hypothesis of polyporic acid formation from 2 mole of phenylpyruvic acid as formulated by Read, Vining and Haskins[8] it is proposed that phenylalanine forms a reactive methylene group by being bound to pyridoxal phosphate as shown (Fig. 2b). Condensation with phenylpyruvic acid (by nucleophilic attack of a carbanion formed from the methylene group) would give a quinoneimine analogue of polyporic acid (Fig. 2d), and oxidative cleavage of this intermediate pyridoxal-bound pulvinamide (Fig. 2e). The latter could be an immediate precursor of most or all pulvinic acid derivatives. By hydrolysis of the imine bond one would get free pulvinamide (plus pyridoxal phosphate) which in turn could give rise to pulvinic acid. Pulvinic acid (plus pyridoxaldimine phosphate, giving pyridoxal phosphate by hydrolysis and dehydration) might also arise by hydrolysis of the acid imide bond, calycin by a corresponding hydrolysis following hydroxylation and vulpinic acid by methanolysis. Finally, transamidation could lead to the formation of the amino acid conjugates of pulvinic acid.

The hypothesis offers the advantage of simplicity and details which can readily be tested by further experiments. It does not exclude the alternative mechanism involving polyporic acid as an intermediate. However, the smooth incorporation of polyporic-^{14}C acid into pulvinic acid derivatives[3] does not necessarily mean that polyporic acid is normally involved in their biosynthesis. Free polyporic acid is detected only in about 1 per cent of the species which produce pulvinic acid derivatives and could arise as a by-product via hydrolysis of a bound quinoneimine intermediate.

Unless condensation involves 2 mole of pyridoxal-bound phenylalanine, feeding of 1-^{14}C- or 2-^{14}C-labeled phenylpyruvate to the lichen under slightly nitrogen-deficient

[6] C. F. CULBERSON, *Chemical and Botanical Guide to Lichen Products*, The University of North Carolina Press, Chapel Hill (1969).
[7] K. MOSBACH, *Biochem. Biophys. Res. Commun.* **17**, 363 (1964).
[8] G. READ, L. C. VINING and R. H. HASKINS, *Can. J. Chem.* **40**, 2357 (1962).

conditions could give pulvinic acid derivatives in which all or much of the activity resides in the enol carbons and none or little in the carboxyl carbons, depending on the extent of dilution by transamination. Another obvious test concerning the formation of pulvinamide would be a double-labelling experiment with phenylalanine-^{14}C-^{15}N. Furthermore, the possible involvement of protein-bound pyridoxal phosphate can readily be studied, and this is currently under investigation.

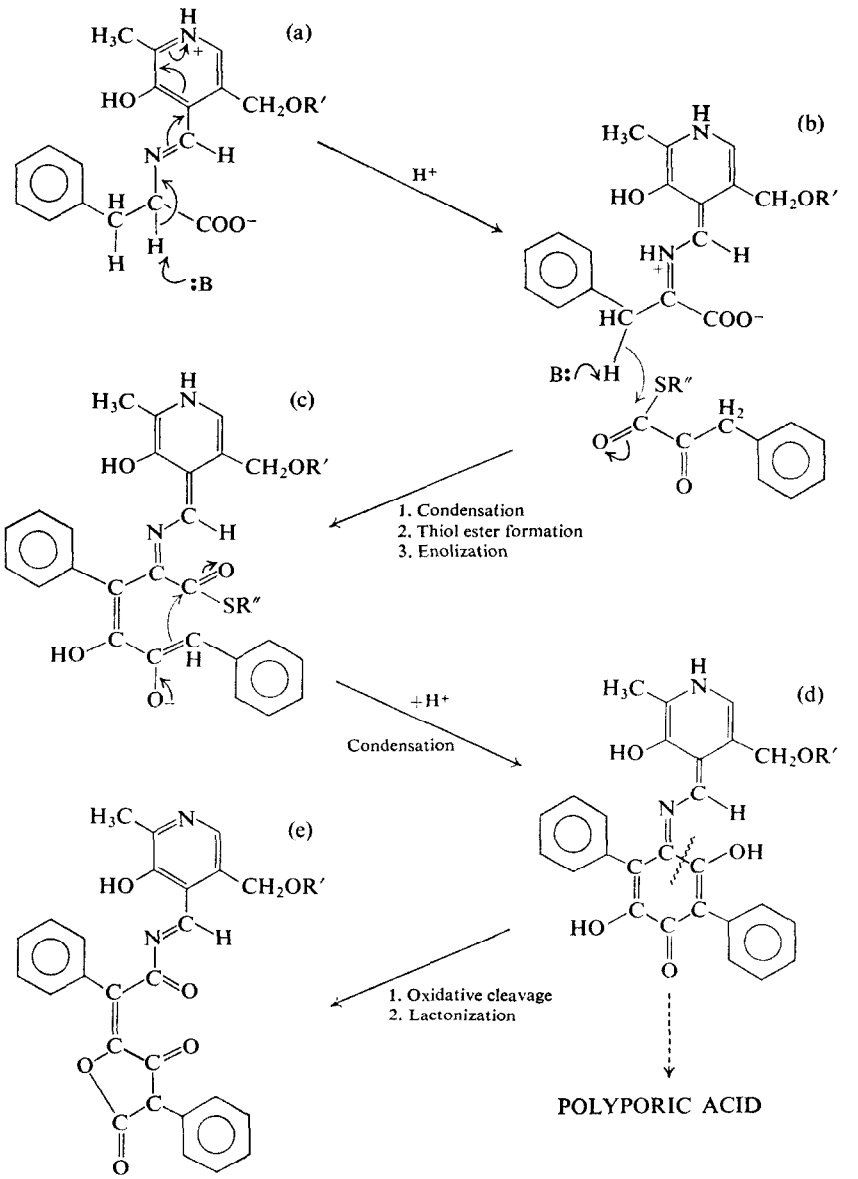

FIG. 2.

R′ = Phosphate residue bound to protein. R″ = Coenzyme A or an equivalent activating residue.

EXPERIMENTAL

Mass spectra (70 eV) were recorded on a Bell & Howell C.E.C. 21–110 instrument using a direct introduction probe.

Isolation of Pulvinamide

Aq. Na_2CO_3 (5%) and NaOH (5%) fractions of ether extracts of *Pseudocyphellaria crocata* were chromatographed on silicic acid with CH_2Cl_2.[1] Pulvinamide was obtained from the fraction containing calycin. Crystallization of this crude material from acetone gave calycin, m.p. 248–249°, and the mother liquors gave a further crop of calycin. The remaining material was chromatographed on preparative TLC plates (silica gel G by Merck and benzene–dioxane–HOAc, 90:25:4). A yellow band (R_f 0·65–0·75) was recovered and crystallization from $CHCl_3$ gave pulvinamide (27 mg from 1·6 kg air-dried lichen), m.p. 220–221° dec, *m/e* 307·0841, 290·0572 (*m** 274); τ (acetone-d_6) 1·85 (4H), 2·50 (6H), 2·67 (1H, exchangeable against D_2O), 2·90 (1H, exchangeable against D_2O), OH unobserved; ν_{max} (KBr) 3466, 3447, 3360, 3328, 1767 and 1670 cm^{-1}; λ_{max} ($CHCl_3$) 377 and 276 mμ (ϵ 11,600 and 12,500).

Synthesis of Pulvinamide

Pulvinamide was synthesized by analogy to the procedure of Frank *et al.*[9] A moderately slow stream of ammonia was bubbled through a boiling solution of $CHCl_3$ (250 ml) containing pulvinic dilactone (2·9 g). After refluxing for 1 hr during which appreciable quantities of the product had precipitated in crystalline form, the solvent was evaporated and the product purified by chromatography on activated silicic acid with $CHCl_3$ as an eluant. Recrystallization of the main colored fraction from $CHCl_3$ afforded pure pulvinamide (3·0 g), m.p. 220–221° dec (mixed m.p. with the natural sample without depression; found: C, 70·73; H, 4·44; N, 4·51; O, 20·32; $C_{18}H_{13}NO_4$ requires: C, 70·36; H, 4·23; N, 4·56; O, 20·85%); mass, NMR, i.r. and u.v. spectra were identical to those of the natural compound.

Sublimation of Pulvinamide

This was carried out at 185–200° (bath temperature) and 2 mm Torr. Some pulvinic dilactone, pulvinic acid and other yellow materials were found in the sublimate as well as in the unsublimed residue as decomposition products.

Conversion of Pulvinamide into Pulvinic Dilactone

Pulvinamide (100 mg) was refluxed in Ac_2O (15 ml) causing a color change from orange-yellow to pale yellow within 15 min. After boiling for 1 hr the reaction mixture was cooled whereupon the bulk of the product crystallized. The crystals were collected in a glass-sintered funnel and identified as pure pulvinic dilactone, m.p. 224–225°. More dilactone was recovered from the filtrate which had been passed into ice water and allowed to evaporate under a stream of air. Combined yield: 98% of theory.

Preparation of O-Methylpulvinamide

Synthetic pulvinamide (300 mg) was dissolved in acetone (300 ml) and treated with an excess of freshly prepared CH_2N_2 for 0·5 hr. After the addition of a few drops of HOAc, the solvent was evaporated and the residue recrystallized from methanol to give large yellowish flakes contaminated by tiny reddish needles. Most of the crystals could be separated by suspending them in $CHCl_3$. The flakes formed a floating layer upon shaking and could be decanted. A second crystallization from methanol gave 240 mg of creamy white flakes, m.p. 218° dec (found: C, 71·24; H, 4·67; N, 4·48; O, 19·61; $C_{19}H_{15}NO_4$ requires: C, 71·03; H, 4·67; N, 4·36; O, 19·93%); *m/e* 321, 278 (*m** 240·8), 263 (*m** 248·8), 235 (*m** 210), 207 (*m** 182·3), 178, 145, 129, 117, 89; τ (acetone-d_6) 2·22 (4H), 2·50 (6H), 2·60 (1H, exchangeable against D_2O), 3·15 (1H exchangeable against D_2O), 6·19 (3H); ν_{max} (KBr) 3411, 3302, 3253, 3194, 1742 and 1659 cm^{-1}; λ_{max} ($CHCl_3$) 330, 269 and 262·5 mμ (ϵ 22,300, 13,100 and 14,000).

Natural pulvinamide (5·0 mg), dissolved in ether (100 ml) and similarly treated with CH_2N_2, gave *O*-methylpulvinamide in good yield (4·8 mg) after only one crystallization from methanol, m.p. 217–218° dec, *m/e* 321·0994 ($C_{19}H_{15}NO_4$ 321·1001), 278·0941 ($C_{18}H_{14}O_3$ 278·0943). Its identity with the fully synthetic *O*-methylpulvinamide was further established by a mixed m.p. (undepressed) and by NMR, i.r. and u.v. spectrometry.

Acknowledgements—I am grateful to my colleagues Drs. S. H. Safe and L. C. Vining for valuable chemical advice, Messrs. D. J. Embree and J. Van Ingen for mass spectra and NMR spectra respectively, and Mr. R. Brown for excellent technical help.

[9] R. L. FRANK, S. M. COHEN and J. N. COKER, *J. Am. Chem. Soc.* **72**, 4454 (1950).