Phytochemistry, 1975, Vol. 14, pp. 1351–1354. Pergamon Press. Printed in England.

A NEW DICARBOXYLIC AMINO ACID FROM SEEDS OF *PENTACLETHRA MACROPHYLLA*

EDDIE I. MBADIWE

Agricultural Research Council's Food Research Institute, Colney Lane, Norwich, NR4 7UA, England

(*Received* 24 *October* 1974)

Key Word Index—*Pentaclethra macrophylla*; Leguminosae; 3-hydroxyaspartic acid; penmacric acid; pyrrolidonecarboxylic acid.

Abstract—The title compound was isolated and shown by various physical and chemical techniques to be a new pyrrolidonecarboxylic acid.

INTRODUCTION

At a Symposium of The Phytochemical Society held at Swansea in 1974, G. Dardenne [1] and the author [2] read papers about work, done independently, which resulted in the isolation and structural elucidation of the same novel amino acid from seeds of *Pentaclethra macrophylla* Benth. The trivial name "penmacric acid" (PMA) was proposed [2]. By mutual agreement, and with editorial co-operation, it was arranged that papers describing the work should be published simultaneously. The author thanks Dr. Dardenne for making available the text of the preceding paper [3], which the present paper is intended to complement.

RESULTS AND DISCUSSION

In the course of work connected with various nitrogenous constituents of seeds of *Pentaclethra macrophylla*, the aqueous phase from a Bligh and Dyer extraction [4] was examined for free amino acids etc. by automatic ion-exchange chromatography [5]. An unusual zone emerged just ahead of aspartic acid and in the same position as *erythro*-DL-3-hydroxyaspartic acid [6–8]. This coincidence extended to electrophoretic behaviour of the novel substance at pH 6 and was misleading for a considerable length of time.

The amino acid responsible for the novel zone was isolated and subjected to preliminary characterization as described under Experimental. The purification was monitored at each step by high-voltage filter-paper electrophoresis at pH 6.

The new acid PMA showed no appreciable UV absorption. Its electrophoretic behaviour was consistent with that of a dicarboxylic monoamino acid, and electrophoresis of the *N*-carboxy derivative in alkaline solution [9] confirmed the presence of a single amino group. Under the conditions for hydrolysis of proteins (6 M-HCl for 24 hr at 110°), followed by evaporation to dryness and ion-exchange chromatography [5], PMA gave 5 ninhydrin-reactive zones. One of these could have been unchanged PMA. These zones presumably represent components of the hydrolysis–relactamization mixture studied in detail by Dardenne and colleagues [1, 3].

Various volatile derivatives of an early crude sample of PMA were prepared and subjected to MS study. Methyl esterification followed by acetylation gave a product whose MS contained a possible molecular ion at *m/e* 272. Ions representing feasible losses of 32 and 43 m.u. were present at *m/e* 240 and *m/e* 229. Major ions were observed at *m/e* 213, 171 and 111, which were interpreted as consecutive losses of -COOMe, CH_2CO and MeCOOH. The mass of M^+ was measured as 272·10021 ($C_{11}H_{16}N_2O_6$ = 272·10083). Therefore 2 Me ester groups and possibly only 1 acetyl group were expected in the molecule. Deuterioacetylation gave a product whose MS showed a molecular ion at *m/e* 275, proving that only 1 acetyl group

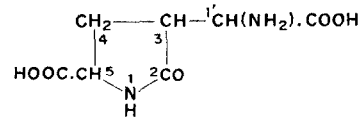
Table 1. Low-resolution mass spectrum of penmacric acid

m/e	Rel. int.*	*m/e*	Rel. int.*	*m/e*	Rel. int.*
25·0	0·32	63·0	0·32	102·0	0·25
26·0	2·46	64·0	0·38	106·0	0·25
27·0	9·03	65·0	1·26	107·0	0·25
29·0	6·06	66·0	2·08	108·0	1·20
30·0	8·65	67·0	4·23	109·0	0·63
31·0	0·51	68·0	18·83	110·0	1·20
33·5	0·32	69·0	2·34	111·0	8·97
35·0	0·51	70·0	12·63	112·0	1·71
36·0	5·43	71·0	0·25	113·0	1·52
37·0	0·82	72·0	0·95	114·0	0·95
38·0	1·52	73·0	1·33	122·0	0·38
39·0	8·21	74·0	7·58	123·0	0·57
40·0	2·53	75·0	0·57	128·0	0·32
41·0	14·28	76·0	0·25	129·0	36·96
41·5	0·32	78·0	1·39	130·0	1·39
42·0	5·94	79·0	0·51	135·0	2·53
43·0	3·85	80·0	0·57	136·0	0·32
44·0	100·00	82·0	2·59	139·0	5·18
45·0	5·31	83·0	23·37	140·0	1·71
46·0	1·71	84·0	55·65	141·0	0·82
50·0	0·51	85·0	2·78	142·0	0·32
51·0	0·88	86·0	1·01	156·0	5·50
52·0	1·83	87·0	0·57	157·0	63·04
53·0	3·54	88·0	0·44	158·0	5·05
54·0	7·39	94·0	1·01	159·0	0·25
55·0	7·01	95·0	2·91	184·0	7·26
56·0	49·53	96·0	8·65	185·0	0·76
57·0	1·90	97·0	1·07	186·0	0·19
60·0	1·07	100·0	1·33		
61·0	0·25	101·0	0·32	202·0	0·19

* Relative intensity as % of intensity of base peak (*m/e* = 44).

had been introduced. Major ions at *m/e* 216, 172 and 112, consistent with losses of -COOMe, CD_2CO and MeCOOH, supported earlier findings. Permethylation of the acetylated methyl ester introduced 2 further methyl groups giving a product M = 300·13210 ($C_{13}H_{20}N_2O_6$ = 300·13212). LRMS again showed the 3 losses described above. The original compound was assumed to have MW 202 and, when pure PMA was available, it gave LRMS (Table 1) showing a very low intensity M^+ at *m/e* 202. More abundant ions at M − 18 and M − 45 supported this assumption, also indicating the presence of -COOH. Recognition of the low-mass fragmentation pattern (*m/e* 129, 84, 56 and 41) as being consistent with that of pyrrolidonecarboxylic acid (PCA) [10] (Annex 2 of Supplementary Publication) was helpful in leading to the proposed structure.

Elementary analysis of PMA was consistent with 2–3% hydration of $C_7H_{10}N_2O_5$. All the evidence so far indicated that PMA was a monoaminodicarboxylic acid. It was next subjected to study by PMR. The 90 MHz PMR spectrum of PMA in D_2O solution showed 5 non-exchangeable hydrogens which, from first-order analysis of their spin–spin couplings (confirmed by spin-decoupling experiments), could be allocated to 4 adjacent carbon atoms in the manner -CH-CH_AH_B-CH-CH-. The protons on the terminal carbon atoms of this unit at δ 4·32 (triplet, $J_{average}$ = 8·5 Hz) and δ 4·07 (*d*, J = 6·5 Hz) are both strongly deshielded and could be assigned to hydrogens on carbon atoms each α- to an amino acid function. The former of these signals is very close in chemical shift to that of the α-proton of PCA, which resonated at δ 4·40. The magnitude of the geminal coupling constant (J_{AB} = −13 Hz) between the 2 protons H_A and H_B of the methylene group was also consistent with a five-membered cyclic structure. The deduced structure (**1**) (no stereochemistry) has been numbered as in ref [3].



Penmacric acid (**1**)

On potentiometric titration of PMA to pH 6 with KOH, the apparent pK was 3·4 (cf. 3·34 for PCA [11]). Changes of PMR chemical shifts for H-1′ and H-5 were respectively −0·16 ppm and −0·19 ppm, suggestive that the main ionization change was 5-COOH [12]. On treating PMA with one mol. prop. HCl (pH → 2·0), changes of chemical shifts of H-1′ and H-5 were respectively + 0·31 ppm and + 0·13 ppm, suggestive that the main ionization change was at 1′-COOH, typical of the pK range for α-amino acids [12, 13].

Strecker degradation of PMA with *N*-bromosuccinimide [14] yielded one mol. prop. of CO_2, suggesting a carboxyl group α- to the free amino group. It had been intended to convert the resulting 3-aldehydo-PCA by Wolff–Kishner reduction [15] to a 4-methylproline [16, 17] or 4-methylglutamic acid [18] derivative, with a view to establishing relative configuration at C-3, but the recent Belgian work [1, 3] has made this unnecessary.

Meanwhile, some evidence had been obtained bearing on absolute configurations at C-1′ and C-5.

On adding 1 mol. prop. of HCl to PMA $\Delta[\alpha]_D$ was positive ($[\alpha]_D^{26\cdot4}$ +21° (H_2O, C= 0·013) i.e. in the same sense as the change on acidification reported in the preceding paper [3] and suggestive of L-(S) configuration by the Clough–Lutz–Jirgensons rule. Further, Dr. A. F. Drake of the Chemistry Dept., King's College, University of London, kindly studied the circular dichroism of the $N^{1'}$-2,4-dinitrophenyl derivative [cf. 10 (Supplementary Publication, Annex 3)], which likewise suggested L-(S) configuration at C-1'. On titrating PMA with KOH to pH 6, $[\alpha]_D$ changed from +11 to −12°, whereas, with a partially racemized specimen of L-PCA, $[\alpha]_D$ changed from −8 to −18° on neutralization (cf. 19). This seemed to indicate L-(S) configuration also at C-5, giving still further support to the absolute configurations proposed in the preceding paper [3].

EXPERIMENTAL

Materials. Beans were obtained commercially in Nigeria. Reagents and reference compounds were commercial products.

Isolation and preliminary characterization of PMA. Coarsely ground bean endosperm (100 g fr. wt) was cold-extracted with 1·5 l monophasic [4] $CHCl_3$–MeOH–H_2O; the extract was then rendered diphasic [4] and the aq. phase charged to a column (180 ml) of Dowex 50W-X8 (H^+ form). After washing the column well with H_2O, 2 successively smaller columns of Dowex 50W-X8 (H^+) were attached below it and displacement [20] was effected with 0·5 M–NH_3. PMA-containing fractions were ahead of and overlapping with aspartic acid. They were pooled and charged to a column (25 ml) of Dowex 1-X8 (AcO^-). After washing with H_2O, gradient elution was applied, using AcOH (8 M) through a mixing chamber (38 ml). [21, 10 (Supplementary Publication, Annex 1)] PMA emerged when the effluent was 2–3 M-AcOH. PMA-containing fractions were pooled and evaporated to dryness *in vacuo* below 40°; PMA crystallized readily from H_2O (solubility at 30° was approx. 25 mg/ml). It was recrystallized to constant optical rotation ($[\alpha]_D^{27°}$ + 11° (H_2O, c = 0·016)). (Found (Pascher, Bonn): C, 40·53; H, 4·85; N, 13·64; O, 40·21%. $C_7H_{10}N_2O_5$ requires: C, 41·59; H, 4·99; N, 13·86; O, 39·57%). Yield was 0·03% of endosperm dry matter. An extract from a different batch of beans had PMA content (estimated by ion-exchange chromatography) 0·18% of endosperm dry matter (cf. [3]).

High-voltage filter-paper electrophoresis [22]. Migration rates are expressed relative to those for aspartic acid in the same electrolyte. In aq. C_5H_5N (2·5% v/v)- AcOH (0·3% v/v) (pH approx. 6) PMA migrated anionically at 0·84; in aq. AcOH (8·7% v/v) -89% (w/v) HCO_2H (2·5% v/v) (pH approx. 1·8), cationically at 0·79. In 0·1 M-NaOH (CO_2 added) [9], PMA gave 2 ninhydrin-staining zones (anionically at 0·82 and 1·00); aspartic acid gave an additional faster zone (*N*-carboxyaspartic acid) at 1·14.

Derivatization. The dimethyl ester HCl was prepared from PMA with methanolic HCl [23]; then acetylated or deuterioacetylated with Ac_2O–C_5H_5N [10]; the product was permethylated [24]. All these derivatives were examined as the crude products. PMA was $N^{1'}$-2,4-dinitrophenylated and the product purified by column chromatography (R value ~0·1) [25].

Ion-exchange chromatography. A Beckman 120C Amino Acid Analyzer was equipped with a column (55 × 0·9 cm) of Beckman UR-30 ion-exchange resin, which was eluted with the recommended pH 3·25 buffer (90 min) and pH 4·25 buffer (110 min) [5]. Column temp was maintained at 56° and buffer flow rate at 58 ml/hr. Eluted zones were detected by reaction with ninhydrin as recommended [5]. Elution time for PMA relative to that for aspartic acid was 0·87; its ninhydrin colour factor relative to D-norleucine was 0·98; its ninhydrin $E_{440\,nm}/E_{570\,nm}$ ratio was 0·16. The hot-HCl treatment of PMA gave zones having elution times (rel. to aspartic acid) 0·69, 0·77, 0·86, 0·95 and 1·45.

MS. MS were obtained using the direct-insertion probe at source temp. 200° and ionization energy 70 eV. Accurate mass measurements were made at a resolving power of 10,000 (10% valley definition).

NMR. PMR spectra were measured in D_2O solns after repeated evaporations *in vacuo* with D_2O. Chemical shifts are expressed in ppm from an internal standard of the Na salt of DDS [13]. Coupling constants in Hz: t = triplet; d = doublet. PMA: 4·32, t, J_{4_A5} = 8, J_{4_B5} = 9, H-5; 4·07, d, $J_{1'3}$ = 6·5, H-1'; 3·10, sextet, $J_{1'3}$ = 6·5, J_{34_A} = 9·0, J_{34_B} = 9·5, H-3; 2·75, octet, J_{34_A} = 9·0, J_{4_A5} = 8, $J_{4_A4_B}$ = −13, H-4_A; 1·99, octet, J_{34_B} = 9·5, J_{4_B5} = 9, $J_{4_A4_B}$ = −13, H-4_B. Potentiometric titrations were done using a glass-electrode assembly. Resulting solutions were suitably conc *in vacuo* before polarimetry and transfer to D_2O.

Acknowledgements—The guidance and help of Prof. R. L. M. Synge is gratefully acknowledged. Thanks are due to Messrs J. Eagles, K. R. Parsley and R. Self for MS; to Dr. D. T. Coxon and Mr. K. R. Price for PMR; to Mr. A. M. C. Davies and Miss Mary M. Gilmour for amino-acid analysis; to Dr. A. F. Drake for CD; to Dr. L. Fowden, Dr. J. L. Frahn and Mr. W. M. Laird for help and useful discussions. This work was done while holding a research grant from the Federal Nigerian Government.

REFERENCES

1. Dardenne, G. (1974) *Proc. Phytochem. Soc.* in *Phytochemistry.*
2. Mbadiwe, E. I. (1974) *Proc. Phytochem. Soc.* in *Phytochemistry.*
3. Welter, A., Jadot, J., Dardenne, G., Marlier, M. and Casimir, J. (1975) *Phytochemistry* (in press).
4. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911.
5. Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Analyt. Chem.* **30,** 1190.
6. Hamilton, P. B. (1963) *Analyt. Chem.* **35,** 2055.
7. Wilding, M. D. and Stahmann, M. A. (1962) *Phytochemistry* **1,** 241.
8. Inatomi, H., Inukai, F. and Murakami, T. (1971) *Chem. Pharm. Bull.* **19,** 217.
9. Frahn, J. L. and Mills, J. A. (1968) *Analyt. Biochem.* **23,** 546.
10. Couchman, R., Eagles, J., Hegarty, M. P., Laird, W. M., Self, R. and Synge, R. L. M. (1973) *Phytochemistry* **12,** 707.
11. Okinaka, C. (1927) in *The Sexagint, Being a Collection of Papers Dedicated to Prof. Y. Osaka,* pp. 27–59. Kyoto Kagaku Gakushi-Kwai, Kyoto.
12. Nakamura, A. (1965) *Nippon Kagaku Zasshi* **86,** 780.
13. Roberts, G. C. K. and Jardetzky, O. (1970) *Adv. Protein Chem.* **24,** 447.
14. Chappelle, E. W. and Luck, J. M. (1957) *J. Biol. Chem.* **229,** 171.
15. Todd, D. (1948) *Org. Reactions* **4,** 378.

16. Burroughs, L. F., Dalby, S., Kenner, G. W. and Sheppard, R. C. (1961) *Nature (Lond).* **189,** 394.
17. Dalby, J. S., Kenner, G. W. and Sheppard, R. C. (1962) *J. Chem. Soc.* p. 4387.
18. Blake, J. and Fowden, L. (1964) *Biochem. J.* **92,** 136.
19. Vl. Staněk (1912) *Z. Zuck Ind. Böhm.* **37,** Okt. S.1.
20. Partridge, S. M. (1948) *Biochem. J.* **44,** 521.
21. Hulme, A. C. and Wooltorton, L. S. C. (1958) *J. Sci. Fd Agric.* **9,** 150.
22. Bailey, J. L. (1962) *Techniques in Protein Chemistry*, p. 40. Elsevier, Amsterdam.
23. Senn, M., Venkataraghavan, R. and McLafferty, F. W. (1966) *J. Am. Chem. Soc.* **88,** 5593.
24. Eagles, J., Laird, W. M., Self, R. and Synge, R. L. M. (1974) *Biomed. Mass Spectrom.* **1,** 43.
25. Portugal, A. V., Green, R. and Sutherland, T. M. (1963) *J. Chromatog.* **12,** 183.