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AMINO ACIDS OF *CAESALPINIA TINCTORIA* AND SOME ALLIED SPECIES

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Key Word Index—*Caesalpinia tinctoria*; Caesalpinioideae; 3-hydroxymethylphenylalanine; 4-hydroxy-3-hydroxymethylphenylalanine; baikiain; chemotaxonomy.

Abstract—Two previously unreported amino acids have been found to form major constituents of seed of *Caesalpinia tinctoria*; they have been characterized as 4-hydroxy-3-hydroxymethylphenylalanine and 3-hydroxymethylphenylalanine. The seed also contains a large amount of the imino acid baikiain. The amino acid content of seeds of this species is compared with that for 12 other species of *Caesalpinia* and 14 species in closely related genera.

INTRODUCTION

FOR MANY years azetidine-2-carboxylic acid (A-2-C) was thought to occur exclusively in the Liliaceae and closely related families. However, it was isolated recently from seedlings of the legume *Delonix regia*,[1] and in a restricted survey of some legumes of the same group (group 5, of the sub-family Caesalpinioideae, according to Hutchinson's classification)[2] it was shown to occur in one other species, *Peltophorum pterocarpum*.[1] The survey revealed several unusual amino acids as constituents of species assigned to this group.

Seed of *Caesalpinia tinctoria* contained two obvious unusual components, and during the separation of these compounds a further unusual amino acid was detected. This paper describes the isolation and identification of these compounds. One of the components, baikiain, has been isolated previously from *Baikiaea plurijuga*,[3] which Hutchinson classifies in group 3 of the sub-family Caesalpinioideae. The other two amino acids are previously unreported and have been identified as 4-hydroxy-3-hydroxymethylphenylalanine and 3-hydroxymethylphenylalanine.

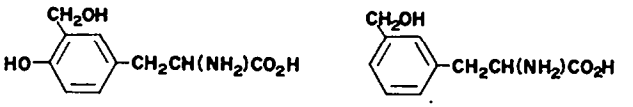
The survey of the amino acid contents of the group of legumes which includes *Caesalpinia* and *Delonix* has been extended and the results of this survey are also given.

RESULTS AND DISCUSSION

The imino acid baikiain and the amino acids 4-hydroxy-3-hydroxymethylphenylalanine (I) and 3-hydroxymethylphenylalanine (II) were isolated by a combination of chromatographic techniques. Baikiain was tentatively identified by the position on two-dimensional chromatograms, and by the characteristic colour of the chromophore given with ninhydrin. This identification was confirmed by comparison of the IR spectrum of the isolated material

[1] M-L. SUNG and L. FOWDEN, *Phytochem.* **8**, 2095 (1969).
[2] J. HUTCHINSON, *The Genera of Flowering Plants*, Clarendon Press, Oxford (1964).
[3] F. E. KING, T. S. KING and A. S. WARWICK. *J. Chem. Soc.* 3590 (1950).

with that obtained using an authentic sample of baikiain, isolated from *Baikiaea plurijuga*, and with the spectral data in the literature.[4,5]



(I) (II)

Elemental analysis of I indicates an empirical formula of $C_{10}H_{13}NO_4$. MS showed a MW of 211 and, therefore, this also represents the molecular formula. The high carbon/hydrogen ratio suggested that I possessed either a polyunsaturated aliphatic skeleton or an aromatic ring. Aromatic character was implied from the UV spectrum of I, which contained a main absorption peak at 277 nm. The aromatic nature of I had been indicated earlier when its ready absorption on to charcoal had been noticed; this characteristic was used ultimately in its isolation.

Hydroxy derivatives of phenylbutyrine represent compounds compatible with the molecular formula of I, but all available compounds of this type showed higher chromatographic mobilities than I in all solvents used. The chromatographic behviour of I, however, closely resembled that of some substituted phenylalanines. Various authentic compounds of this type were reduced by catalytic hydrogenation (Pt/H_2), and the products sampled at intervals; repeatable patterns emerged. A simple phenyl ring, as in phenylalanine, was easily saturated to the cyclohexyl derivative, whilst a phenolic hydroxyl group required more prolonged reduction to give the same product; during the reduction of tyrosine two intermediates were formed. An hydroxyl group present in a second aliphatic substituent (e.g. a hydroxymethyl group as in 4-hydroxymethylphenylalanine), however, was even more difficult to reduce; its presence seemed also to retard the rate of saturation of the phenyl ring: therefore several intermediates could be detected. The products of reduction of I were examined over a 5-hr time course; ten chromatographically distinct intermediates were observed, suggesting a combination of aliphatic and aromatic hydroxyl groups: two hydroxyl groups are compatible with the molecular formula.

The final product of reduction of I behaved identically, on paper chromatograms, with 2-, 3- and 4-methylcyclohexylalanines, prepared by catalytic hydrogenation of the respective ring-methyl substituted phenylalanines; these three isomeric amino acids were inseparable in all the solvent systems tested. However, the elution profiles, obtained from the three methylcyclohexylalanines, using the amino acid autoanalyser, were characteristically different. The profile from the reduction product of 4-methylphenylalanine contained two peaks, the first being larger than the second; reduced 3-methylphenylalanine gave three peaks under these conditions, two of equal size, followed by a larger final peak. The profile given by reduced 2-methylphenylalanine contained two broad peaks which were not coincident with any of the peaks obtained from the 3- and 4-substituted derivatives. Reduction of methylphenylalanines could yield a mixture of *cis*- and *trans*-forms of the saturated products (boat and chair forms of the cyclohexyl ring are also possible), and the presence of double or multiple peaks in the autoanalyser profiles is attributed to such possible configurational isomers. The elution profile for the final reduction product of I had three peaks

[4] R. M. ZACHARIUS, J. F. THOMPSON and F. C. STEWARD, *J. Am. Chem. Soc.* **76**, 2908 (1954).
[5] N. GROBBELAAR, J. K. POLLARD and F. C. STEWARD, *Nature, Lond.* **175**, 703 (1954).

of the same relative magnitude as those obtained after reduction of 3-methylphenylalanine: in a mixed run, these peaks were exactly coincident.

The NMR spectrum showed that there were three aromatic protons, of which two were adjacent and one was isolated, with a weak *meta* coupling. A two proton singlet suggested a $-CH_2OH$ group, and there were resonance peaks which showed a structure identical to the alanyl residue of phenylalanine and tyrosine. Examination of the UV spectra for I revealed a positive wavelength shift, from 277 to 297 nm on addition of alkali, indicative of a phenolic hydroxyl group.[6] These various points of evidence leave only 4-hydroxy-3-hydroxymethylphenylalanine as a possible formula for I.

The coexistence of II with I in the same plant, and their similar absorption behaviour upon charcoal, suggested that II might have an aromatic structure related to that of I. Their comparative behaviour on paper chromatograms was compatible with the idea that II contained one less hydroxyl group than I; II was more mobile than I in solvents containing acetic acid, whilst their mobilities were identical in basic solvents, e.g. ethyl acetate–pyridine–water. The UV spectrum of II had a main absorption peak at 262 nm with shoulders at 265 and 271 nm. Addition of alkali did not affect the spectrum indicating the absence of phenolic hydroxyls.

During catalytic hydrogenation of II fewer intermediate products were detected than in the case of I, and complete reduction was effected more easily. The final reduction product of I and II behaved identically on paper chromatograms, whilst the profile of peaks eluted from the autoanalyser was again similar to that obtained after reduction of 3-methylphenylalanine.

The NMR spectrum was consistent with these data, and indicated four aromatic protons. It also showed resonance peaks attributable to a $-CH_2OH$ group and an alanyl residue as in I. The position of substitution of the hydroxymethyl group on the aromatic ring was established by oxidation of II using a Kiliani reagent.[7] The oxidation product, a carboxylphenylalanine, was electrophoretically identical with authentic 3-carboxyphenylalanine, and distinct from 4-carboxyphenylalanine (prepared identically by oxidation of authentic 4-hydroxymethylphenylalanine).

It has been shown, in *Reseda odorata*, that 3-carboxyphenylalanine and 3-carboxytyrosine are derived from shikimic acid, and that the aromatic carboxyl group is derived from the carboxyl group of shikimic acid.[8] 3-Carboxyphenylalanine is present in small quantities in the seed of *C. tinctoria*, which suggests that II arises by a shikimate pathway whose final stages involve the reduction of 3-carboxyphenylalanine. Similarly, I could be derived from 3-carboxytyrosine, although this compound was not detected on normal two-dimensional chromatograms of *C. tinctoria* seed extracts. Since the levels of I and II fall on germination of the seed, this hypothesis presumably could be tested satisfactorily only during the phases of seed growth and maturation.

The amino acid content of dry seeds of 13 species of *Caesalpinia*, and of 14 other species assigned to group 5 of the Caesalpinioideae,[2] are shown in Table 1; attention is confined to unusual, non-protein amino acids. The genus *Caesalpinia* contains about 280 species[2] showing a wide range of form and so the sample examined is small. The majority of species analyzed contained one or more representatives of a group of γ-substituted glutamic acids. The extremely large accumulations of γ-methylglutamic acid and γ-methyleneglutamic acid

[6] G. SCHNEIDER, *Naturwissenschaften* **44**, 422 (1957).
[7] J. W. PELLETIER and D. M. LOCKE, *J. Am. Chem. Soc.* **87**, 761 (1965).
[8] P. O. LARSEN, *Biochim. Biophys. Acta* **141**, 27 (1967).

in seed of *C. bonduc* and *C. ferrea*, respectively, were particularly noteworthy. However, such γ-substituted derivatives of glutamic acid were not detected in seed extracts of *C. tinctoria* and *C. sepiaria*. Seeds of most of the *Caesalpinia* species shown in Table 1 have been germinated, and the amino acid composition of seedlings determined. In general, germination was accompanied by marked increases in the content of the various γ-substituted glutamic acids, and these compounds became notable constituents of *C. tinctoria*

TABLE 1. THE OCCURRENCE OF CERTAIN NON-PROTEIN AMINO ACIDS IN SEEDS OF VARIOUS SPECIES ASSIGNED TO THE CAESALPINIOIDEAE

Species	γ-Methyleneglutamic acid	γ-Methylglutamic acid	γ-Ethylidineglutamic acid	γ-Ethylglutamic acid	γ-OH-γ-methylglutamic acid) / β-OH-γ-methylglutamic acid)	Pipecolic acid	5-OH-pipecolic acid	4-OH-pipecolic acid	Baikiain	3-OH-proline	*m*-Carboxyphenylalanine	3-Hydroxymethylphenylalanine	4-OH-3-hydroxymethyl-phenylalanine	Azetidine-2-carboxylic acid
Caesalpinia bonduc	w	s*	m	w	t									
C. coriaria		m					w							
C. ferrea	s*	m	t								t			
C. gilliesia	t	t								m				
*C. jayabo***		w	m	t										
C. major		w	m	t	t	m	t							
C. minax†		s	w	t										
C. morsei†			m				m							
C. nuga	t	w												
C. pulcherrima	t	t												
C. sappem														
C. sepiaria			t			w		t			w			
C. tinctoria						s	m	m	s*		w	s	s*	
Burkea africana							m							
Bussea massaiensis	m						m	m		m				s*
Delonix regia										s*				
Erythrophloeum africanum		w				m			w					
E. guiniense		m												
Gleditsia amaphoides		m			s		w	w						
G. triacanthos		w	t		t	w	s							
Parkinsonia aculeata										m				w
Peltophorum africanum							m	w						
P. inermi		t					m							
P. linnaei	s	m	t				t							
Pterolobium stellatum														
Schizolobium parahybum														
Stachyothyrus standii							w							
Wagatia spicata				s										

** Considered as a misnomer applied variously to *C. bonduc* and *C. nuga*.[9]

† Considered to be representatives of a single species.[9]

Relative concentrations of amino acids: s, strong; m, moderate; w, weak; t, trace. Asterisk used to indicate compound was principal component of free amino acid pool.

[9] J. P. M. BRENAN, Kew Herbarium, personal communication.

seedlings. Similar increases in the number and the concentrations of the γ-substituted glutamic acids were observed during germination of seeds of many of the other genera listed in Table 1.

Caesalpinia tinctoria was unique among the *Caesalpinia* spp. examined by its content of baikiain and the two new aromatic amino acids described in the first part of this paper. It is then of interest to recall that morphologically, *C. tinctoria* is also distinct from the other *Caesalpinia* spp. examined; it has a fleshy pod contrasting sharply with the flattened, dry pods of other species of *Caesalpinia*, and is also characterized by paired spines on the adult shoot, that are not found elsewhere in the genus.

Table 1 shows that azetidine-2-carboxylic acid was detected as a seed constituent of only two species examined: it formed the principal component of the soluble nitrogen fraction from seed of *Bussea massaiensis*. The imino acid was produced in considerable quantities during germination of some other species (*Delonix regia*, *Peltophorum inerme*, *P. africanum*, *Schizolobium parahybum*), and the amount present in *Parkinsonia aculeata* increased markedly during early seedling growth.

EXPERIMENTAL

Chromatographic procedures. PC was used to compare I and II, and their reduced forms, with other amino acids. At least two of the following solvents were used for each comparison: *n*-BuOH–HOAc–H_2O (BAW) (90:10:29), 75% (w/v) phenol–NH_3, EtOAc–pyridine–H_2O (2:1:2, upper phase), 2-methylbutan-2-ol–HOAc–H_2O (20:1:20, upper phase). A Technicon amino acid autoanalyser, operating on a standard 21 hr programme,[10] was used to compare the elution profiles given by the reduced amino acids I and II, and by reduced methylphenylalanines. Exact coincidence of peaks was checked by runs on mixed samples.

Isolation of amino acids I *and* II. Aromatic amino acids were isolated from the seed (1·7 kg) extract by absorption on to a column of charcoal activated with 5% (v/v) HOAc;[11] non-aromatic amino acids were not absorbed. I and II, together with some phenylalanine and tyrosine, were eluted by a phenol–HOAc solution, used to give a linear gradient of concentrations from zero to 5% (w/v) phenol, 20% (v/v) HOAc. I and II were separated by preparative PC in BAW as above (detection by UV). After elution, non-cationic materials were removed using Dowex 50, the absorbed amino acid being displaced with N–NH_3. The eluates were dried and triturated with MeOH to give pale yellow solids. Purities were checked chromatographically. II contained a very small amount of I, and I contained a small amount of an impurity attributable to spontaneous oxidation. Yields for I and II were 630 and 220 mg respectively; for I [$\alpha\ _D^{20}$ −36° (*c*, 1 in H_2O), and $[\alpha]_D^{20}$ −4° (*c*, 0·5 in 5 N HCl) indicating L-configuration; solutions of II were too deeply coloured to allow accurate determination of optical rotations.

Isolation and identification of baikiain. Baikiain was isolated from fractions not absorbed on to charcoal, by preparative PC (as above), and was crystallized from aq. EtOH at −5°. The IR spectrum (Nujol) was identical with that obtained from authentic baikiain from *Baikiaea plurijuga*.

Other procedures. UV spectra of amino acids (0·1–0·5 mg/ml, pathlength 1 cm) were determined in H_2O (3 ml) after the addition of 0·3 ml N–NaOH containing 0·3 mg Na_2SO_4 to the solution.

Hydrogenations were carried out at laboratory temp. and pressure by bubbling a slow stream of H_2 through a solution of the amino acid (2 mg/ml) in 5% (v/v) HOAc, in the presence of Adam's Pt catalyst. In the case of I, which was difficult to reduce completely, hydrogenation was continued for 5 hr. The Kiliani reagent was prepared by dissolving 0·53 g CrO_3 in 0·8 g H_2SO_4 and 4 ml H_2O. The reagent (1 ml) was added to 1 mg of II dissolved in 1 ml HOAc at 0°. The mixture was allowed to attain room temp., and remain for 1 hr, before the reaction was stopped by the addition of MeOH. The solution was basified (saturated $Ba(OH)_2$), the precipitated $BaSO_4$ removed, and the oxidized amino acid isolated by absorption on to and elution from Dowex 1 anion-exchange resin and Zeocarb 225 cation-exchange resin sequentially. The products were compared with natural 3-carboxyphenylalanine, and with the product obtained after a similar oxidation of 4-hydroxymethylphenylalanine, by paper electrophoresis at pH 3·4[12] (100 V/cm, 170 min). The electrophoretic mobility of the oxidation product from II (16·0 cm towards anode) was identical with that of 3-carboxyphenylalanine, whereas 4-carboxyphenylalanine (by oxidation of 4-hydroxymethylphenylalanine) was less mobile (12·8 cm towards anode) and gave a green chromophore with ninhydrin.

[10] *Technicon Manual*, AAA-1. Technical Corporation, New York (1967).
[11] J. M. PARTRIDGE, *Biochem. J.* **44**, 521 (1949).
[12] P. J. PETERSON and L. FOWDEN, *J. Chromatog.* **48**, 575 (1970).

Survey of the amino acid content of related spp. Ground seed material and seedlings (when available) were extracted in 75% EtOH and the amino acid fraction separated using small Zeocarb 225 columns. Two-dimensional chromatograms were developed using phenol–NH_3 as the first solvent, followed by BAW. Wherever strong chromaphores in the position of γ-aminobutyric acid were noticed, a further chromatogram was developed using EtOAc–pyridine–H_2O as the second solvent, because γ-aminobutyric acid and II are inseparable in the first solvent combination. Compound I moves near alanine on combined phenol–NH_3, BAW chromatograms.

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