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ELUCIDATION OF STRUCTURE AND BIOSYNTHESIS OF ACACIPETALIN

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Abstract—The structure of acacipetalin isolated from *Acacia sieberiana* var. *woodii* has been revised, primarily on the basis of NMR spectra. Biosynthetic studies on the compound show that L-leucine is the most effective precursor of the aglycone. The isolation of a second cyanogenic glycoside closely related to acacipetalin is reported.

INTRODUCTION

A cyanogenic glycoside was first isolated from the South African plants *Acacia hebeclada* and *A. sieberiana* var. *woodii* by Steyn and Rimington [1, 2]. These authors presented arguments to show that this compound, which they named acacipetalin, possessed a previously undescribed structure (**1**). As the aglycone could reasonably be derived from leucine, studies were undertaken to determine the degree of incorporation of leucine as well as other amino acids in an attempt to clarify the biosynthesis of this unique compound.

Recent studies with cyanolipids from *Ungnadia speciosa* (Sapindaceae) [3] have revealed other cyanogenic compounds with "aglycone" structures (**2**) which could be considered derived from leucine* We have been careful to compare the chemical and spectral data from *Acacia sieberiana* var. *woodii* with that of these cyanogenic lipids. As a result of these comparisons we have concluded that the originally proposed structure is incorrect. Evidence for the corrected structure (**3**) is primarily spectral in nature; the aglycones of similar materials have been shown to be rather difficult to manipulate chemically [3, 4]. Our feeding studies have indeed confirmed that leucine is incorporated into acacipetalin. In addition, a second cyanogenic glycoside has been detected in *A. sieberiana* var. *woodii*.

RESULTS AND DISCUSSION

Mass spectra, NMR data, and enzymic hydrolysis established that the major cyanogenic compound isolated in this study is the monoglycoside to which Rimington assigned the name acacipetalin. The field desorption MS [5] of the compound possessed a strong (M + 1) ion at 260 *m/e*. Accurate mass measurements with electron impact techniques (70 eV) confirmed these results and substantiated the empirical formula: $C_{11}H_{17}NO_6$. Rimington [2] had demonstrated that the sugar of acacipetalin is glucose by hydrolysis followed by preparation of the osazone.

Analysis of NMR spectra of acacipetalin revealed, however, that the structure of the aglycone (**1**) proposed by Steyn and Rimington is untenable. These spectra indicated the presence of

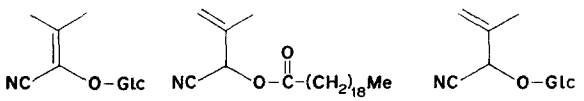
* In another Sapindaceous plant, *Koelreuteria paniculata*, we have demonstrated that the cyanolipids are derived from leucine. This work is the subject of another paper now in preparation.

Table 1. A comparison of NMR data for the TMS ether of acacipetalin and for the cyanolipid of *Ungnadia speciosa*

	Multiplicity		Chemical shift (δ)*	
	Glycoside	Lipid	Glycoside	Lipid
Methyl protons	*br s*	*br s*	1·78 (3)	1·89
Cyanohydrin proton	*s*	*s*	5·29 (1)	5·79
Vinyl protons	*s*	*s*	4·86 (1)	5·17
	s	*s*	5·04 (1)	5·33
Glucose protons	*m*	—	3·0–3·5 (~4)	—
$-CH_2-OTMS$ of glucose	*d* (*J* 3 cps)	—	3·62 (2)	—
Anomeric proton	*d* (*J* 7 cps)	—	4·35 (1)	—

* NMR spectra measured on a 100 mc Varian HA-100 instrument in CCl_4 (acacipetalin–TMS ether) and $CDCl_3$ (cyanolipid).

vinyl protons (4·86, 5·04 δ) as well as a methyl group at 1·78 δ. Comparison of the spectral data of this compound with that of the cyanolipid of *Ungnadia speciosa* (**2**) and a spectrum of glucose–TMS allowed the assignment of protons as shown in Table 1. Double resonance techniques indicated that the methyl, vinyl and cyanohydrin protons are coupled (<1 cps) and are part of the same allylic system. Irradiation of the methyl proton absorption (1·90 δ) sharpened the absorptions at 5·27, 5·42 and 5·36 δ, demonstrating that these groups of protons are weakly coupled to the methyl protons. Conversely, irradiation at 5·27 δ sharpened the peak corresponding to the methyl protons (1·87 δ).



(1) (2) (3)

Enzymic hydrolysis of acacipetalin yielded methacrolein which was identified by NMR spectral examination of the 2,4-dinitrophenylhydrazone (δ values of absorptions in $CDCl_3$: 2·07 δ, three protons, methyl group; 5·4 δ and 5·57 δ, one proton each, vinyl protons; 7·79 δ, one proton, cyanohydrin proton; 7·4–9·3, aromatic protons). The presence of a smaller amount of isobutyraldehyde 2,4-dinitrophenylhydrazone was suggested by a doublet centered at 1·28 δ. Thus we conclude that acacipetalin possesses structure (**3**).

The TMS–ether of acacipetalin is readily separated by GLC from those of glucose and the second cyanogenic glycoside (Compound A) isolated in this study. This technique was used to determine qualitatively the purity of various fractions encountered in this work. Cyanide analyses [6] indicated that acacipetalin and Compound A are present in *A. sieberiana* var. *woodii* in the approximate ratio of 3:1. The structure of Compound A, which we believe to be dihydro-acacipetalin, will be reported separately.

Biosynthetic studies

In order to find suitable conditions for biosynthetic studies on acacipetalin, the concentration of cyanogenic material was examined during the germination of seedlings. Although the level of cyanogen increased somewhat, the amount of cyanogenic material was low. The concentration in young, vigorously growing shoots was higher and preliminary experiments subsequently showed these tissues to be suitable for biosynthetic studies.

The effectiveness of various compounds to serve as precursors of acacipetalin was determined by measuring the ^{14}C contained in the HCN released when the cyanogenic glucoside was hydrolyzed with linamarase. This approach was chosen because, in contrast to other glycosides which have been studied, the methacrolein expected on hydrolysis of acacipetalin is moderately unstable. Indeed, Rimington's original papers [1, 2] did not clarify the nature of the products of acacipetalin hydrolysis except for glucose and HCN. Although methacrolein was obtained as the 2,4-dinitro-

Table 2. Incorporation of ^{14}C-labelled amino acids and [1-^{14}C]-acetic acid into the nitrile group of acacipetalin

Compound administered	HCN isolated		
	μmol	dpm/μmol	% Incorporation
L-[U-^{14}C]-leucine	1·26	8200	0·125
L-[U-^{14}C]-leucine	1·50	6040	0·108
L-[U-^{14}C]-isoleucine	0·94	0	0
L-[U-^{14}C]-isoleucine	1·00	0	0
L-[U-^{14}C]-valine	1·35	577	0·008
L-[U-^{14}C]-valine	1·35	31	0·0004
L-[U-^{14}C]-phenylalanine	—	Trace	Trace
L-[U-^{14}C]-tyrosine	—	0	0
[1-^{14}C]-acetic acid	1·68	0	0
[1-^{14}C]-acetic acid	0·96	0	0

The amount of ^{14}C available (in the α-carbon atom of the amino acids administered) for conversion to HCN ranged from 6×10^6 to 12×10^6 dpm.

Table 3. Incorporation of ^{14}C-labelled amino acids and [2-^{14}C]-acetic acid into the nitrile group of acacipetalin

Experiment	Compound administered: Label	Radioactivity (dpm)	^{14}C-convertible to HCN* (dpm)	HCN isolated: (μmol)	HCN isolated: (dpm)	Incorporation (%)
1	L-[U-^{14}C]-leucine	$7{\cdot}4 \times 10^7$	$1{\cdot}2 \times 10^7$	3·4	$4{\cdot}1 \times 10^3$	0·034
	L-[U-^{14}C]-leucine	$7{\cdot}4 \times 10^7$	$1{\cdot}2 \times 10^7$	3·1	$3{\cdot}7 \times 10^3$	0·031
2a	[2-^{14}C]-acetic acid	$4{\cdot}1 \times 10^7$	$4{\cdot}1 \times 10^7$	3·1	$3{\cdot}3 \times 10^3$	0·008
	[2-^{14}C]-acetic acid	$4{\cdot}2 \times 10^7$	$4{\cdot}1 \times 10^7$	3·7	$3{\cdot}3 \times 10^3$	0·008
2b	[2-^{14}C]-acetic acid after prefeeding L-valine	$4{\cdot}2 \times 10^7$	$4{\cdot}2 \times 10^7$	3·9	$5{\cdot}3 \times 10^4$	0·13
	[2-^{14}C]-acetic acid after prefeeding L-valine	$4{\cdot}1 \times 10^7$	$4{\cdot}1 \times 10^7$	4·0	$4{\cdot}9 \times 10^4$	0·12

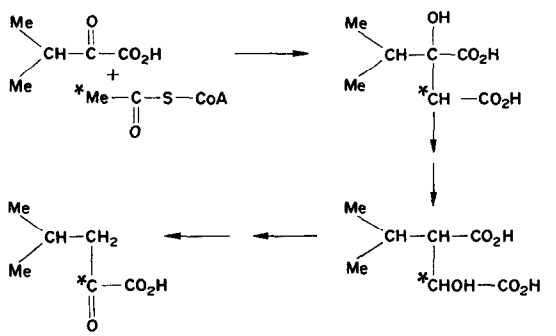
* Amt. of ^{14}C in leucine administered, or produced according to Scheme 1, that is available for conversion to HCN.

phenylhydrazone during the characterization of the glycoside reported above, the smaller quantities of aglycone involved in the feeding experiments did not permit isolation of this derivative.

Table 2 shows that the amino acid leucine is the most effective precursor of the HCN obtained on hydrolysis of acacipetalin; of the four other amino acids previously shown [7] to be precursors of the aglycones of cyanogenic glucosides, only valine gave rise to some labeled HCN. That result was examined further and is discussed below. [1-^{14}C]-acetic acid, which might possibly serve as a precursor of the isoprenoid structure found in acacipetalin, did not give rise to labeled glycoside.

The ability of L-[U-^{14}C]-valine to label acacipetalin was examined further in the experiment described in Table 3. Butler and Shen [8] have shown that valine can be converted to leucine in flax seedlings, presumably by the route involving the conversion of keto-valine to keto-leucine demonstrated for microorganisms by Umbarger and associates [9]. In this sequence (Scheme 1) keto-valine condenses with acetyl-CoA to form α-isopropylmalic acid. This compound in turn is converted to β-isopropylmalic acid; decarboxylation and oxidation yields keto-leucine.

In the course of these reactions, C_1 and C_2 of the acetyl moiety of acetyl-CoA becomes the C_1 and C_2 of leucine while four of the five carbon atoms of valine complete the leucine skeleton. More specifically, the methyl group of acetyl-CoA becomes the C_2 of leucine; however, that carbon atom, which gives rise to HCN [7], should *not* become labeled from L-[U-^{14}C]-valine administered to the plant. The fact that [U-^{14}C]-valine did give rise to the [^{14}C]-HCN (Table 2) suggests that randomization of label into leucine has occurred via acetyl-CoA.



Scheme 1. The formation of the keto-acid analog of leucine from the keto-acid analog of valine and acetyl-CoA.

To examine if the scheme might be operating, acacia shoots were first administered unlabeled L-valine to increase the pool size of that amino acid; then [2-^{14}C]-acetic acid was administered and the result was compared with shoots which had been fed either [U-^{14}C]-leucine or [2-^{14}C]-acetic acid without any pretreatment. Table 3 (Experiment 1) shows that L-[U-^{14}C]-leucine gave rise to [^{14}C]-HCN, although not as extensively as observed in Table 2. When the shoots were administered [2-^{14}C]-acetic acid (Experiment 2a), a low level of incorporation into the nitrile group was observed. However, the amount of incorporation was increased about 15-fold when the shoots were first prefed with L-valine (Experiment 2b). Indeed, the

incorporation exceeded that observed for L-[U-^{14}C]-leucine.

EXPERIMENTAL

Plant material. Seed of *Acacia sieberiana* var. *woodii*, obtained from the Botanical Research Institute, Pretoria, South Africa, were germinated and several young trees grown in a greenhouse. Voucher specimens of the plants are deposited in the University of Illinois herbarium.

Isolation and purification of glycosides. Acacia sieberiana leaves (1 kg) were blended in 4 l. hot 80% EtOH. The soln was strained through cheesecloth, centrifuged, and the supernatant taken to dryness. The residue was dissolved in 80 ml 50% EtOH, 300 ml of a MeOH–$CHCl_3$ soln added and the mixture shaken for 30 sec. H_2O (96 ml) was then added and the mixture again shaken for 30 sec. $CHCl_3$ (96 ml) was then added to form two phases [10], the mixture shaken again for 30 sec and then left for 90 min while the phases separated. The top layer (aq MeOH) was saved. The $CHCl_3$ layer was then re-extracted with 300 ml 50% MeOH; the aq. MeOH phase removed, combined with the first fraction, and taken to dryness.

The residue was dissolved in a minimum amount of 50% EtOH and placed on a Si-gel column (40 × 6 cm i.d.) prepared from a propanol slurry. Elution was started with propanol and 200 ml fractions were collected. After 8–10 fractions, when the eluate became less colored, the eluting solvent was changed to 90% propanol. Again, when the eluate became less colored, the elution solvent was changed first to 80% propanol and then to 70% propanol. The fractions collected were tested for cyanogenic glycosides by placing aliquots (1 ml) in screw-cap vials (4·5 × 1·0 cm i.d.); the solns were evaporated under N_2, a few drops of linamarase obtained from flax seed [11] in 0·1 M phosphate buffer, pH 6·8 added and any cyanide released detected by picrate paper [12]. Under these conditions the major cyanogenic compound subsequently shown to be acacipetalin eluted in 90% propanol while a second, unknown cyanogenic compound (Compound A) appeared in 80% propanol.

The fractions containing acacipetalin were combined, as were those containing Compound A, taken to dryness and each re-chromatographed on the Si-gel column using propanol–H_2O. After elution, detection, combination and evaporation to dryness, the 2 cyanogenic compounds were then further purified by chromatography on Whatman 3 MM paper.

Samples (0·5 g) of the partially purified glycosides in aq. MeOH were streaked on sheets of Whatman 3 MM paper (46 cm wide). The paper was developed in 2-butanone–Me_2CO–H_2O (15:5:3) until the solvent front had moved about 40 cm. Two strips, 2 cm wide, were cut from the center of the chromatogram. One strip was sprayed with aq. $AgNO_3$ [13] to visualize the sugars. The other strip, extending from the origin to the solvent front, was cut transversely into approximately 20–40 pieces, each 2 cm wide. Each of these small pieces in turn was placed in a vial; a few drops linamarase were added, and any cyanide released detected by picrate paper. From the $AgNO_3$ treatment and cyanide test, the areas of the chromatogram which contained the glycosides were identified.

The glycosides were eluted from paper in a minimum amount of 50% EtOH, the eluates were concentrated to a small vol. and streaked on sheets of Whatman 3 M for re-chromatography in the 2-butanone–Me_2CO–H_2O system. After the second chromatography, the glycosides were eluted and taken to dryness. The last traces of H_2O were removed in a vacuum desiccator over P_2O_5.

Enzymatic hydrolysis of acacipetalin. Glycoside (73 mg), isolated by PC, was hydrolyzed by stirring overnight with β-glucosidase (10 mg) at room temp. The mixture was then steam distilled and a fraction collected at 85–100°. This fraction was treated with 2,4-dinitrophenylhydrazine in diethylene glycol dimethyl ether to yield an orange–red ppt. The NMR spectrum of this ppt. (in $CDCl_3$) corresponded to that of authentic methacrolein 2,4-dinitrophenylhydrazone. 2,4-dinitrophenylhydrazine and a small amount of isobutyraldehyde 2,4-dinitrophenylhydrazone. The mother liquor contained only 2,4-dinitrophenylhydrazine.

GLC procedures. TMS ethers of the cyanogenic glycosides and glucose were prepared by treating dried samples with trimethylchlorosilane in pyridine for 30–60 min. Chromatography of the derivatives obtained was performed on a chromatograph equipped with flame ionization detector and a glass column (1·8 m × 2 mm i.d.) packed with 3% SP-2250 on Supelcoport 80–100 mesh. Carrier gas, helium, 30 ml/min; temp program, 200° for 1 min, ΔT of 5°/min, final temp 300°, hold for 2 min. Injector temp, 260°; detector temp, 350°.

Feeding experiments. Young shoots (1–2 g) at the tips of rapidly growing branches were utilized in the studies on biosynthesis. The ends of the shoots were cut under H_2O and immersed in aq. solns of the various compounds that were examined as precursors. After a period of metabolism, usually 24 hr, during which the solutions were replenished with H_2O, the shoots were ground in liquid N_2 and extracted in boiling 80% EtOH. After filtration, the extracts were taken to dryness, dissolved in a minimum of 50% EtOH and chromatographed, first in 2-butanone–Me_2CO–H_2O (15:5:3) and then in PrOH–H_2O (4:1). The cyanogen was located on the chromatogram as described above by cutting a strip (2 cm) off the edge of the chromatogram, dividing the strip into 20–30 small sections and testing with linamarase and picrate paper.

The area of the chromatogram containing the cyanogen was eluted with 50% EtOH, the eluate taken to dryness and the residue dissolved in H_2O. An aliquot of this soln was then hydrolyzed with linamarase, and the HCN released trapped in 1 N NaOH [14]. The NaCN–NaOH was then analyzed for its cyanide content [6] and for ^{14}C and from these data the sp. act. of the HCN released and the percent incorporation of precursor into the cyanogenic glycoside was calculated.

Because the alkaline NaOH–NaCN causes Triton–toluene counting mixture to luminesce [15, 16] the counting procedure was carefully standardized as follows: the NaOH–NaCN produced from the 1 N NaOH trap was diluted appropriately so that the NaOH concn was 0·1 N; 1·7 ml of this soln was then added to 10 ml of Triton–toluene counting mixture [16] in a counting vial and vigorously shaken to produce a clear soln. The vials were then placed in the dark at room temp. for 7 days before counting; under these conditions the counts due to the base-induced luminescence in the ^{14}C-window of the Beckman scintillation spectrometer (Model LS-230) were negligible.

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