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TETRAPHYLLIN B AND EPITETRAPHYLLIN B SULPHATES: NOVEL CYANOGENIC GLUCOSIDES FROM *PASSIFLORA CAERULEA* AND *P. ALATO-CAERULEA*

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Key Word Index—*Passiflora caerulea*; *P. alato-caerulea*; Passifloraceae; cyanogenic glycoside; cyanogenic glycoside sulphate; tetraphyllin B; tetraphyllin B sulphate; NMR.

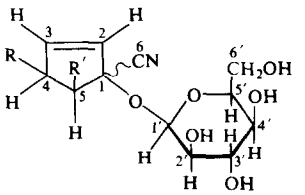
Abstract—An epimeric mixture of tetraphyllin B-4-sulphate and epitetraphyllin B-4-sulphate was isolated from *Passiflora caerulea* and *P. alato-caerulea*. The structure was determined largely by means of the ^{1}H and ^{13}C NMR spectra of the sulphates and their corresponding tetra-acetate derivatives.

INTRODUCTION

Members of the Passifloraceae have long been recognized as being cyanogenic [1–3]. A number of cyanogenic compounds have been isolated and chemically characterized except for those of the genus *Passiflora*. Fruits of *Passiflora* are commonly eaten in the tropics and are known variously as passion fruit, granadilla and maracuja. It is not generally recognized, however, that immature fruits of the genus are often quite toxic and some (e.g. those of *P. adenopoda* DC.) on ingestion have proven fatal [5]. The leaves, stems, and the arils of immature seeds are the most strongly cyanogenic parts of most species [5]. The roots of several taxa are known to contain alkaloids and have been used medicinally. Chemical–plant–insect interactions have been recognized between several passifloraceous species and butterflies of the genus *Heliconius* [4].

Several members of the genera *Passiflora* and *Adenia* have previously been reported to contain cyclopentenoid cyanogenic glycosides, primarily on the basis of R_f values from PC [6]. The study cited reported the major cyanogenic glycoside of *Adenia lobata* (Jacq.) Engl., *P. adenopoda* DC., *P.* × *allardii* Lynch, *P. caerulea* L. and *P. suberosa* L. to be gynocardin (**4**), but noted that *P. adenopoda* and *P. suberosa* contained a second glycoside. Another glycoside, deidaclin (**3**), isolated from *Deidamia clematoides* (C. H. Wright) Harms proved to have a non-hydroxylated cyclopentenyl ring [6, 7]. The second cyanogen of *Adenia lobata*, *P.* × *allardii*, and *P. caerulea* coincided in chromatographic properties with deidaclin [6]. The cyanogen of *P. racemosa* Brot. was not identical with gynocardin or deidaclin in chromatographic properties.

Subsequently, two additional cyanogenic glycosides, tetraphyllin A and B (**1**), were isolated from *Tetrapathaea tetrandra* Cheeseman and characterized by spectral and chemical methods [8]. Although one of these, tetraphyllin A, was similar in structure to deidaclin, NMR spectral studies showed that the two compounds are distinct [10] and must be epimers. Tetraphyllin B is probably identical with barterin which was previously isolated from *Barteria fistulosa* Mast. [11]. A mixture of tetraphyllin B and an epimer, epitetraphyllin B was later isolated from *Adenia volkensii* Harms. [12]. We have recently shown the cyanogen of the tuber of *Adenia digitata* Engl. to be



1 R = OH, R′ = H Tetraphyllin B, epitetraphyllin B
2 R = OSO_3^-, R′ = H Tetraphyllin B sulphate epimeric mixture
3 R = R′ = H Deidaclin, tetraphyllin A
4 R = R′ = OH Gynocardin

tetraphyllin B, primarily by NMR spectral methods [33].

Several workers have previously examined the cyanogens of *P. caerulea* [13–16] which has been considered poisonous to livestock [17]. A hybrid of this plant and *P. alata* (Dryand.) Ait., *P. alato-caerulea* Lindl., has apparently not been previously studied.

Although the basic structures are known for the five previously mentioned glycosides, the overall stereochemistry has been determined only for gynocardin [18, 19]. Thus, deidaclin and tetraphyllin A are epimers as are tetraphyllin B and epitetraphyllin B, but the stereochemistry of each member of the pairs is unknown. A cyanogenic glycoside sulphate has previously been reported. Hübel and Nahrstedt isolated the sulphate of cardiospermin from *Cardiospermum grandiflorum* [9].

RESULTS AND DISCUSSION

Data from PC studies indicated that the major cyanogen of *P. caerulea* and *P. alato-caerulea* was a polar compound which was not identical in R_f value to the compounds previously reported from these and related species. Further, materials of *P. caerulea* collected in Buenos Aires, Argentina and those grown in our greenhouse, as well as cultivated *P. alato-caerulea* collected in mid-winter and mid-summer in California all had the same major constituent. In some samples, however, small amounts of less polar compounds were observed which had similar values to those previously reported [6].

When crushed, fresh plant material of *P. caerulea* and *P. alato-caerulea* rapidly liberate copious quantities of cyanide (30–40 μmol/g). Neither methanol, aqueous, nor acetone extracts liberated cyanide spontaneously, indicating that the cyanide occurred as part of a relatively stable molecule. Addition of β-glucosidase enzymes from almond (emulsin) and flax (linamarase) failed to liberate cyanide or liberated it at an extremely low rate. A β-glucosidase preparation made from an acetone powder of fresh leaf material of *P. alato-caerulea* readily liberated cyanide from the extracts. A similar effect was previously reported [6] with gynocardin isolated from *Hydnocarpus wightiana* (Flacourtiaceae) seeds.

In order to monitor the purification and fractionation of crude extracts of the cyanogen of *P. caerulea* and *P. alato-caerulea* it was necessary to use a β-glucosidase preparation prepared from one of these or related plants. The cyanogen proved quite difficult to purify. Chromatography of the cyanogen of *P. alato-caerulea* on Sephadex G-10 followed by cellulose and subsequent final purification on paper yielded samples which still contained traces of impurities, probably sugar derivatives. We discovered that chromatography of samples over cellulose (prepared as in the Experimental) and subsequent PC (Whatman 3 MM) yielded relatively large amounts of clean glycosides. It was necessary to allow the chromatograms to run in this solvent for 7 days, until almost all impurities have been eluted from the sheets. The R_f of the glycoside was *ca* 0.1.

The purified glycoside was hydrolysed in a Warburg flask and assayed for both cyanide and glucose. The sugar was determined to be glucose by the glucose oxidase method [26]. Because the ratio of cyanide to glucose was 1:1 the glycoside had to be a monoglucoside. As it was quite polar, tests to determine the presence of polar functional groups were carried out. A sodium fusion revealed the presence of sulphur in the molecule. Hydrolysis of the glycoside with sulphatase enzymes and subsequent addition of barium hydroxide yielded a dense white precipitate of barium sulphate. Electrophoretic separation of the purified glycoside of *P. alato-caerulea* was conducted at pH 2. At this pH all common phenols and carboxylic acids are protonated and only strongly acidic compounds remain ionic. Movement of the compound toward the cathode indicated that an anion was present [27]. These data and the MW as determined by field desorption mass spectroscopy strongly suggested the presence of a sulphate group in the molecule.

As all known cyanogenic compounds from the Passifloraceae and related families contain a cyclopentenoid moiety [6, 28–34], it was considered probable that this compound also contained this structural feature. This was confirmed by examination of the NMR spectrum of the cyanogen (Table 1). The chemical shifts, multiplicities of peaks and coupling constants of the cyanogen protons were all quite similar to those reported for tetraphyllin B [8, 10] and epitetraphyllin B [12]. The presence of tetraphyllin A, deidaclin and gynocardin, all of which possess cyclopentenoid moieties, was conclusively ruled out by comparison of NMR spectra. The cyanogen possessed two vinyl proton doublets centred at δ6.45 and 6.25, a multiplet at δ5.1, two four-line patterns centred at δ2.89 and 2.38 and peaks which approximate those of glucose (Fig. 1). These peaks corresponded closely to those reported for tetraphyllin B [8]. The fact that the new cyanogen contained sulphate, had a distinct mass spectrum, formed an insoluble TMS ether ($CDCl_3$), failed to elute on GLC under conditions where tetraphyllin B elutes, and had distinct TLC and PC R_f values indicated that it was not tetraphyllin B.

The identity of each proton was established by decoupling experiments. Irradiation of the double doublet at δ 6.25 sharpened the multiplet at δ 5.1 and collapsed the double doublet at δ 6.45 to a singlet. Irradiation of the peak at δ 6.45 caused a similar effect. Irradiation of the multiplet at δ 5.1 yielded sharpened doublets at δ 6.45 and 6.25 and collapsed the four-line patterns (δ 2.89 and 2.38) to doublets. When the spectrum of the cyanogen sulphate was re-run by adding methanol-d_4 and only the amount of D_2O necessary to dissolve the sample, several changes were noted (Fig. 2). The multiplet of H-4 was shifted from δ 5.1 to 5.55. The protons adjacent to the sulphate group in cardiospermin 5-sulphate (δ 4.68) were previously reported to be shifted downfield from those of cardiospermin (δ 4.22) [32]. An additional multiplet appeared between the two vinyl doublets previously observed (at δ 6.25 and 6.45), a situation reminiscent of that observed in previous studies of the TMS ethers of tetraphyllin B and epitetraphyllin B [12]. The position of the H-4 multiplet of tetraphyllin B was unchanged under these conditions and the vinyl protons were still two pairs of apparent doublets. The anomeric proton of

Table 1. ^{1}H NMR spectral data for tetraphyllin B (**1**) and its penta-acetate derivative*, and the tetraphyllin B sulphate epimeric mixture (**2**) and its tetra-acetate derivative*

	1 (D_2O)	**2** (D_2O)	**1** (MeOH-d_4 + D_2O)	**2** (MeOH-d_4 + D_2O)	Penta-acetate **1** ($CDCl_3$)	Tetra-acetate **2** ($CDCl_3$)
H-2	6.46 *dd* (1,6,1)†	6.45 *dd* (1,5.47,0.9)	6.46 *dd* (1,6,1)	6.41 *d* (1,5.9,1.9) 6.53 *dd* (1,5.6,2.2)	6.34 *dd* (1,5.44,2.0)	6.34 *dd* (1,5.2,1)
H-3	6.43 *dd* (1,6,1)	6.25 *dd* (1,5.20,–)	6.43 *dd* (1,6,1)	6.16 *dd* (1,5.7,1.3) 6.39 *dd* (1,5.7,1.7)	6.08 *dd* (1,5.72,0)	6.08 *dd* (1,6.0,0)
H-4	5.11 *m* (1,–,–)	5.12 *m* (1,–,–)	5.11 *m* (1,–,–)	5.66 *m* (1,–,–) 5.59 *m* (1,–,–)	5.74 *m* (1,–,–)	5.74 *m* (1,–,–)
H-5(2)	2.38 *dd* (1,15,4.5)	2.38 *dd* (1,17.22,2.7)	2.38 *dd* (1,15,4.5)	2.46 *dd* (1,15,4,3.9) 2.69 *dd* (1,15.4,7.1)	2.46 *dd* (1,15.20,2.89)	2.47 *dd* (1,15,2.7)
	2.90 *dd* (1,15,6)	2.89 *dd* (1,14.86,6.2)	2.80 *dd* (1,15,6)	2.77 *dd* (1,15.3,3.3)	2.87 *dd* (1,15.49,6.77)	2.88 *dd* (1,15,6.8)
H-1′	4.72 *d* (1,7,–)	4.70 *d* (1,7.7,–)	4.72 *d* (1,7,–)	2.96 *dd* (1,15.2,7.4) 4.32 *d* (1,7.84,–)	4.88 *d* (1,7.71,–)	4.88 *d* (1,7.7,–)
H-5′	3.80 *m* (1,–,–)	3.80 *m* (1,–,–)	3.80 *m* (1,–,–)	4.77 *d* (1,7.89,–)	3.88 *m* (1,–,–)	3.8 *m* (1,–,–)
H-6′	3.92 *m* (1,–,–)	3.95 *m* (1,–,–)	3.92 *m* (1,–,–)	3.73 *m* (2,–,–)	4.23 *m* (1,–,–)	4.23 *m* (1,–,–)
H-2′,3′4′	3.20–3.81 *m* (3,–,–)	3.2–3.80 m (3,–,–)	3.20–3.81 m (3,–,–)	3.88 *m* (2,–,–) 3.2–3.6 *m* (6,–,–)	5.01–5.26 *m* (3,–,–)	5.01–5.26 *m* (3,–,–)

*Acetate peaks occurred at δ 2.0–2.5; integrals were 15 for tetraphyllin B penta-acetate and 12 for the sulphate tetra-acetate.
†Figures in parentheses are: integral value, coupling constant (Hz) J'.

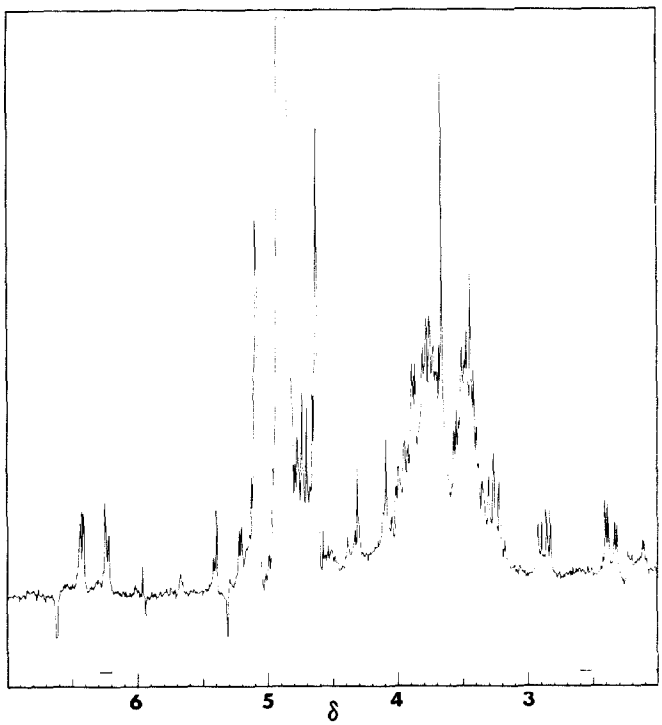


Fig. 1. Tetraphyllin B and epitetraphyllin B sulphates in D_2O.

the cyanogen sulphate was difficult to see in spectra run in either D_2O or MeOH-d_4–D_2O. Spectra of tetraphyllin B in DMSO-d_6 possessed a doublet corresponding to the anomeric proton at δ 4.4 (7.7 Hz). The spectrum of the cyanogenic sulphate had two doublets centred at δ 4.0 and 4.3 demonstrating clearly the presence of an epimeric mixture (Fig. 3).

The chemical shift values of carbons from the proton noise decoupled ^{13}C spectrum of tetraphyllin B [33] and the published data on related compounds [32] permitted assignment of C-1, C-2, C-3, C-5, C-6, C-1′, C-2′, C-4′ and C-6′ (Table 2)(Fig. 4). C-3′, C-5′, and C-4′ were represented by three peaks from δ 74.95 to 77.02. An off-resonance decoupled spectrum indicated that the two vinyl carbons (142.09 and 139.41) were each coupled to one proton; the nitrile carbon (119.18) and C-1 (81.91) were coupled to none; and C-5 and C-6′ were each coupled to two protons [33]. Examination of the proton noise decoupled spectrum of the epimeric sulphate mixture revealed two peaks, corresponding closely in chemical shift, for C-1, C-2, C-3 and C-5. Only one peak was observed for C-1′,C-2′, C-4′ and C-6′.

Samples of the underivatized epimers were difficult to purify. Analysis by TLC and NMR spectroscopy indicated that small amounts of impurities (probably sugar derivatives) contaminated all samples. For this reason and to reduce HDO absorption in the NMR spectra, the cyanogen sulphate was acetylated. The corresponding acetate was purified by TLC (Si gel). Interpretation of the NMR spectra was thereby simplified. Proton integral values for the acetate indicated the presence of four acetate groups and

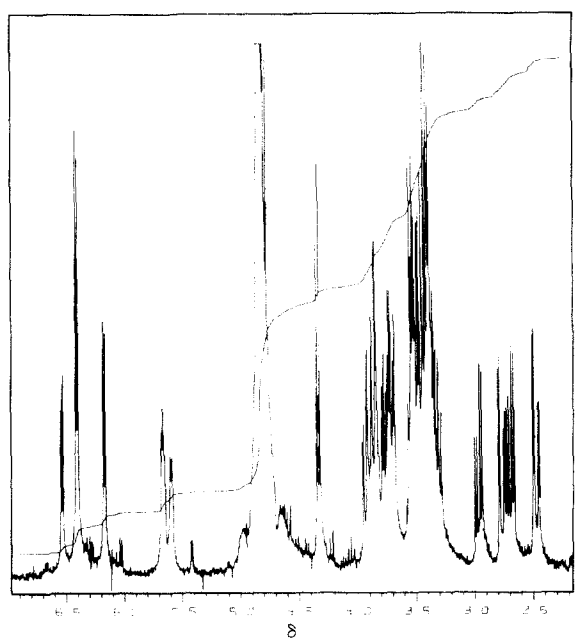


Fig. 2. ^1H NMR of tetraphyllin B and epitetraphyllin B sulphates in D_2O + MeOH-d_4.

confirmed the presence of only one glucose moiety in the molecule. NMR spectral data for the acetate (in $CDCl_3$) are given in Table 1. Two vinyl protons were centered at δ 6.34 and 6.08 (double doublets), H-4

Table 2. ^{13}C NMR spectral data for cyclopentene cyanogens

Compound		C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
Tetraphyllin B (**1**)*	Chemical shift (ppm)	82.91	143.09	132.41	74.95	47.09	120.18	100.53	73.69	77.02	70.23	76.37	61.38
	Configuration upon partial decoupling	*s*	*d*	*d*	*d*	*t*	*s*	*d*	*d*	*d*	*d*	*d*	*t*
Tetraphyllin B sulphate (**2**), epimeric mixture	Chemical shift (ppm)	83.55 82.46	140.44 139.56	134.78 134.15	(76.53)‡	45.11 42.91	120.02	98.58	73.62	(76.53)‡	70.44	(76.53)‡	61.38
	Configuration upon partial decoupling	*d*	*q*	*q*	*q*	*sex.*	*d*	*q*	*q*	*q*	*q*	*q*	*sex.*
Deidaclin†	Chemical shift (ppm)	84.98	143.14	128.73	31.43	37.67	120.92	100.13	73.78	77.08	70.34	76.42	61.47
Gynocardin (**4**)†	Chemical shift (ppm)	85.65	140.91	128.39	78.59	87.36	116.67	100.26	73.69	77.01	70.61	76.43	61.70

*Data from [33].
†Data from [32].
‡Tentative values.

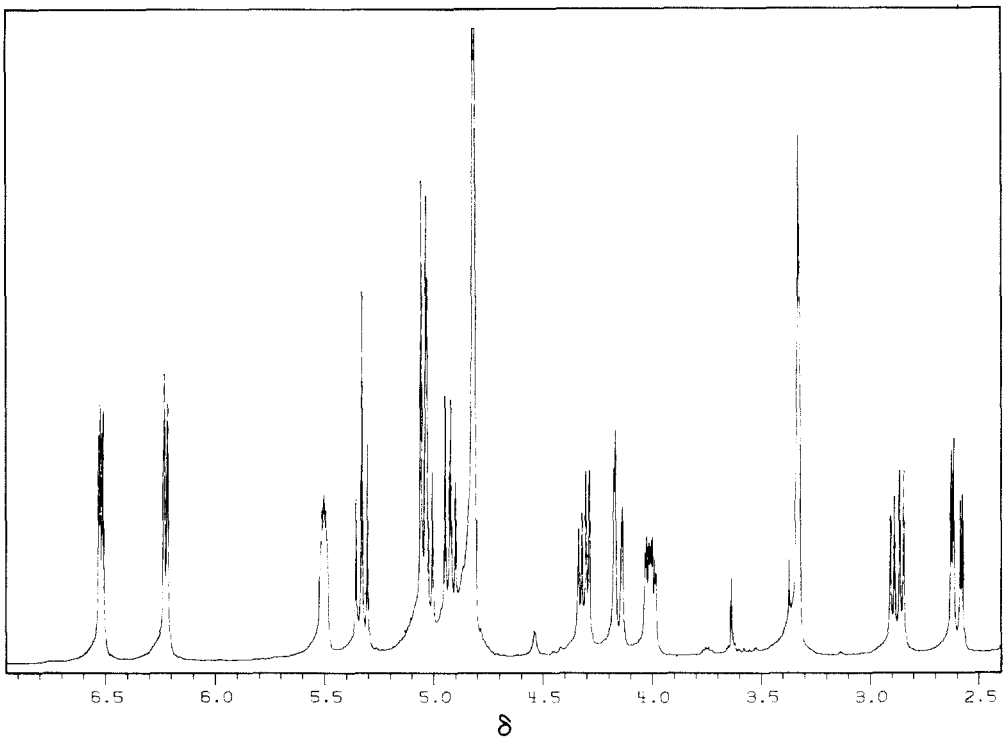


Fig. 3. ^{1}H NMR of tetraphyllin B and epitetraphyllin B sulphate tetra-acetates in D_2O + MeOH-d_4.

appeared at δ 5.74 (multiplet), and the C-5 methylene protons were centred at δ 2.47 and 2.87 respectively (each a four-line pattern). Sugar protons appeared at δ 3.80 (*m*, H-5′), 4.23 (*m*, H_2-6′) and δ4.8 and 5.3 (H-1′ to H-4′, complex pattern). Double resonance studies (measured in MeOH-d_4–$CDCl_3$; chemical shifts of several peaks differ somewhat from those in $CDCl_3$) demonstrated that the H-4 multiplet (δ 5.51) was coupled to the two vinyl protons (δ 6.52 and 6.22) and to the two four-line patterns (centred at δ 2.87 and 2.60). The two four-line patterns were converted to doublets by irradiation at δ 5.51. Assignment of sugar protons was also done by double resonance techniques. The H-5′ multiplet (δ 4.0) was coupled to the two apparent four-line patterns (at δ 4.15 and 4.3), both of which were converted to doublets by irradiation at δ 4.0. Irradiation of the downfield four-line H-6′ proton pattern (δ4.3) converted the four-line pattern at δ 4.15 to a doublet, as well as that at δ 4.0 (H-5′). H-5′ was also coupled to the multiplet at δ5.03. Thus H-4′ must be part of the multiplet of δ 5.03. An apparent triplet at δ 5.3 proved to be coupled to the multiplet at δ 5.04. Irradiation of the multiplet at δ 5.04 changed the apparent triplet at δ5.3 to a singlet, consistent with the behaviour expected for H-2′, H-3′ and H-4′. H-2′ and H-4′ occurred under the multiplet at δ 5.03 as H-3′ was converted to a singlet by irradiation of one position. Three acetate peaks centred at δ 2.08 occurred in a ratio of 1:2:1 and clearly demonstrated the presence of only one sugar.

Although preparation of the TMS derivative appeared to be successful, an entirely suitable solvent for determination of its NMR spectra was not found. The vinyl protons were centred at δ 6.42 and 6.08 and H-4 at 5.52.

As in the case of a previously reported naturally occurring mixture of tetraphyllin B and epitetraphyllin B [12], the corresponding sulphates (in D_2O–MeOH-d_4) appear to differ at the chiral centre bearing nitrile and oxygen and not at the allylic position bearing sulphate. The position of H-4 is identical in the two epimers. Further, the coupling constants of this proton and the two adjacent methylene protons are identical; the chemical shifts of the four pairs of quartets corresponding to the methylene protons differ only slightly. The two quartets centred at δ 2.46 and 2.77 possess a coupling constant of *ca* 4 Hz similar to that of the two *trans*-coupled protons in the TMS ether of gynocardin, known only to have two *trans*-coupled protons [10]. The other two sets of quadruplets (δ2.69 and 2.96) have a coupling constant of 7 Hz which is probably the result of *cis*-coupling. The relative position of the methylene protons and the allylic proton do not appear to differ in the two compounds. In tetraphyllin A and deidaclin, only the chiral centre bearing nitrile and oxygen can differ. Inversion of the centre causes only slight changes in the chemical shifts of the adjacent methylene protons. A shift in the anomeric proton also suggests that it is the chiral centre that differs as inversion of the allylic centre which is more distantly located would not be predicted to produce significant effects. Only 1:1 mixtures of the two compounds are observed. Previous work with tetraphyllin B [12, 31, 33] suggests that it is not easily epimerized, and it is not probable that the corresponding sulphates would epimerize readily. Further, as the chiral centre would be difficult to invert, these two epimeric sulphates almost certainly co-occur in *P. caerulea* and *P. alato-caerulea*.

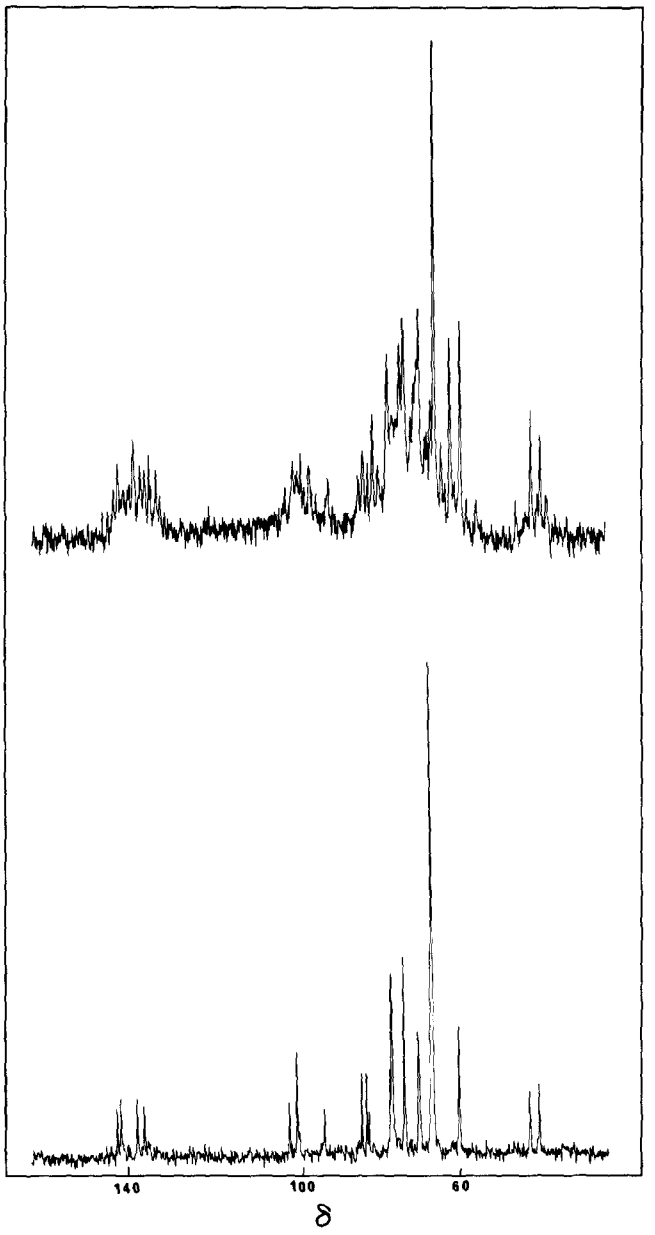


Fig. 4. ORD and SFORD ^{13}C NMR spectra of tetraphyllin B and epitetraphyllin B sulphates in D_2O (int. standard, dioxane).

EXPERIMENTAL

Plant materials. P. caerulea was collected in Buenos Aires, Argentina during November, 1976 (D. S. Seigler and J. Hunziker, s.n., voucher in University of Illinois Herbarium). *P. caerulea* was also grown from commercially available seeds (Applewood Seed Co., Lakewood, Colorado). *P. alato-caerulea* material from Davis, California was obtained from cultivated plants (D. S. Seigler, J. E. Dunn and W. S. Statler, DS-11274, 10 June 1979, voucher in the University of Illinois Herbarium).

Isolation of the glycoside. Leaf material of fresh *P. caerulea* (200 g) was extracted with boiling MeOH for 30 min, filtered and re-extracted twice. The resulting mixture was filtered and concd under vacuum to a syrup. The syrup was extracted with $CHCl_3$ and the combined $CHCl_3$ phases back-extracted with H_2O.

Leaf material of *P. alato-caerulea* (400 g) was ground in cold Me_2CO, filtered and the process repeated. The resulting soln was concd until it was largely aq., extracted with $CHCl_3$ and the resulting aq. phase concd under vacuum to a syrup (13.6 g). A similarly prepared MeOH extract produced a yellow syrup (21.4 g). All $CHCl_3$ washes were tested and proved to be negative for the presence of HCN.

Enzyme preparation. A Me_2CO powder of *P. alato-caerulea* (as prepared above) was dispersed in 0.02 M Pi buffer (pH 6.8). The mixture was stirred (30 min, 0°) and solid material removed by centrifugation. The supernatant was placed in dialysis tubing which had been boiled for 10 min and dialysed against the same buffer. The enzyme preparation was removed and stored at 2°. This enzyme preparation rapidly liberated HCN (determined by the picrate [21, 22] and Feigl–Anger methods [6, 20]) from both the MeOH and Me_2CO extracts of the two species.

Purification of the crude cyanogenic extract. Portions of

the syrup from *P. caerulea* were initially purified by means of a Sephadex G-10 column packed and eluted with H_2O. *Ca* 5–10 ml concd syrup was placed on the column and fractions (5 ml) were collected. Cyanogenic fractions (usually *ca* fractions 15–30) were located by enzymatic hydrolysis of aliquots and the Feigl–Anger method. These fractions were then placed on a column of microcrystalline cellulose (Applied Science)-powdered cellulose (Whatman CF-1) (1:1). Fractions (20 ml) were collected with *iso*-PrOH–*n*-BuOH–H_2O (6:3:1) as eluant. Cyanogenic fractions were located as above, combined and concd under vacuum. These partially purified materials were further purified by PC (Whatman 3 MM), with MeCOEt–Me_2CO–H_2O (15:5:3). The presence of the major cyanogen was determined by removing portions of the chromatogram, desorbing the cyanogen and hydrolysing as above. The active band (R_f 0.1) was eluted with H_2O, MeCOEt concd and re-chromatographed on paper with Me_2CO–H_2O (5:1). The cyanogen (R_f 0.2) was eluted and re-chromatographed in MeCOEt–Me_2CO–H_2O (15:5:3) to yield a light-yellow syrup (0.5 ml).

Syrup of *P. alato-caerulea* was initially purified by passing over a column of cellulose [microcrystalline cellulose (Applied Science)–cellulose powder (Whatman F-1) (1:1) packed in *iso*-PrOH–*n*-butanol–H_2O (6:3:1)]. Cyanogenic fractions were pooled, concd under vacuum and again passed over a cellulose column in Me_2CO–H_2O (5:1). Fractions of 20 ml were collected; fractions 10–40 (of 200), which contained the cyanogenic compounds, were pooled and concd. The resulting syrup was placed on paper (Whatman 3 MM) and developed in *iso*-PrOH–*n*-butanol–H_2O (6:3:1) for 7 days. The cyanogenic material remained at R_f 0.1 whereas most contaminants were eluted from the chromatograms.

TLC of the purified cyanogen. Samples of purified cyanogen were spotted on microcrystalline cellulose TLC plates and developed in MeCOEt–Me_2CO–H_2O (15:5:3) or Me_2CO–H_2O (4:1). These plates were visualized by sequential spraying with $AgNO_3$ (0.6% in Me_2CO), NaOH (0.5 N in EtOH) and aq. 20% $Na_2S_2O_3$ [24]. Spots of impurities (R_f 0.6) rapidly turned black. Cyanogenic compounds were located on TLC plates by spraying with enzyme preparation, and covering with Feigl–Anger test paper. Cyanogenic activity was only found at R_f 0.2. When plates were sprayed with aq. 1% $KMnO_4$ soln a fugitive yellow spot at R_f 0.1–0.2 was observed.

Qualitative determination of S *in the cyanogen.* A Na fusion analysis was carried out on a small sample of the cyanogen (10 mg) from both species [23]. The test proved positive for the presence of S.

Hydrolysis of the cyanogen of P. alato-caerulea *with sulphatase.* A sample of the cyanogen (1 mg) was dissolved in H_2O and sulphatase (0.5 mg, dry, Sigma Chemical Co., Aryl Sulphatase H-1 from *Helix pomatia*) was added. After incubation at 25° for 5 min, three drops of 0.3 N $Ba(OH)_2$ were added. A dense ppt of $BaSO_4$ resulted. Controls with enzyme alone did not yield a ppt.

Electrophoresis of the cyanogen. A sample of the cyanogen (*ca* 20 mg) from both species was placed in the centre of a sheet of paper (Whatman 3 MM, 20 × 40 cm) and the paper sprayed with buffer [HOAc (8%)–HCOOH (2.5%), 1:1]. The sample was subjected to electrophoresis (800 V and greater than 200 mA) for 1.25 hr. The paper was dried and tested for the presence of cyanide as above. Cyanogenic activity was found from the origin to 5 cm toward the cathode.

Quantitative determination of sugar and cyanide. A sample of extract from both species was placed in a Warburg flask and dissolved in Pi buffer (3 ml, 0.1 M, pH 6.8). Enzyme preparation (0.1 ml) was added. 1M NaOH (0.5 ml) was added to the centre well and the flasks were incubated at 37° overnight with shaking. The basic soln was removed and diluted, and a cyanide assay performed by the Lambert method [25]. The basic soln contained 30.5 μmol HCN/ml and the MeOH soluble materials contained 27.4 μmol/ml.

Using the glucose oxidase method [26], a quantitative glucose assay was performed and revealed the presence of 1 mol of glucose per mol of cyanogen (1.4 μmol cyanogen gave 1.3 μmol glucose).

GLC of the cyanogen TMS derivative. The TMS derivative was prepared as previously described. Under conditions by which the TMS derivatives of tetraphyllin A and B, gynocardin and deidaclin were eluted (3% SP-2250 on a Supelcoport SS column, 1.8 m × 2 mm, 220° for 1 min, then programmed at 15°/min to 350°) no response was observed with the cyanogen of *P. alato-caerulea.* A small amount of impurity with identical retention time to tetraphyllin B and epitetraphyllin B was observed.

Preparation of the acetate of the cyanogen. Cyanogen (10 mg) from *P. alato-caerulea* was dried under vacuum and then dissolved in pyridine (0.5 ml). Ac_2O (1 ml) was added and the mixture warmed for *ca* 10 min and the sample was then taken to dryness under vacuum. The solid material was extracted with $CHCl_3$ and the extract concd to a yellow amorphous solid. This material was then purified by prep. TLC on Si gel with $CHCl_3$–C_6H_6–MeOH (40:9:1). Duplicate plates were run. To locate the products one plate was sprayed with dil. H_2SO_4–chromic acid soln and charred for 30 min at 110°. This treatment revealed two spots, at R_f 0.5 and 0.6. The material at R_f 0.5 was established to be the cyanogen acetate by its NMR spectral properties.

MS measurements. The MS was measured on a Varian 311A spectrometer (low resolution). The FDMS of the cyanogen had ions at 365 $[M-1]^+$, 382 $[M+H_2O-1]^+$ and a major fragment at 204 $[M-\text{glucose}+H_2O]^+$.

NMR spectra. Preliminary ^1H NMR measurements were made in each case on a Varian EM-390 instrument at 90 MHz. Purified samples were measured on a Varian HA-220 (220 MHz) or a Nicolet NT-360 (360 MHz) instrument. Decoupling expts were carried out using the latter two instruments. ^{13}C NMR were measured on a Varian FX-60 instrument.

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