PERGAMON

Phytochemistry 51 (1999) 153–157

PHYTOCHEMISTRY

(−)-Phalarine, a furano*bis*indole alkaloid from *Phalaris coerulescens*

Neil Anderton[a], Peter A. Cockrum[a], Steven M. Colegate[a,*], John A. Edgar[a], Kirsty Flower[b], Dale Gardner[c], Richard I. Willing[d]

[a]*Plant Toxins Unit, CSIRO Division of Animal Health, Australian Animal Health Laboratory, Private Bag 24, Geelong, Vic. 3220, Australia*
[b]*Kybybolite Research Centre, P.O. Box 2, Kybybolite, SA 5262, Australia*
[c]*Poisonous Plant Research Laboratory, USDA, Logan ,UT, USA*
[d]*CSIRO Division of Molecular Science, Private Bag 10, Rosebank MDC, Clayton, Vic. 3169, Australia*

Received in revised form 15 October 1998

Abstract

Chemical examination of *Phalaris coerulescens* revealed a new alkaloid, phalarine. Extensive mass spectrometric and NMR spectroscopic examination of phalarine and its monoacetyl and monomethyl derivatives indicated a novel furano*bis*indole structure.
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Keywords: Phalaris coerulescens; Gramineae; indole alkaloids; phalarine; furano*bis*indole

1. Introduction

Perennial grasses of the genus *Phalaris*, such as reed canary grass (*P. arundinacea*) and Toowoomba canary grass (*P. aquatica*), can be valuable pasture components (Cheeke, 1998). However, ingestion of the grass by livestock has resulted in poisoning episodes characterized by acute or chronic central nervous signs or by sudden death (Anderton, Cockrum, Walker, & Edgar, 1994; Bourke, 1994). Despite an extensive plant breeding program directed at producing low-toxicity cultivars of perennial *Phalaris* spp. (Oram, 1970), cases of poisoning still occur (Kennedy, Cregan, Glastonbury, Golland, & Day, 1986; Unpublished data, 1997). The plant breeding programs have been aimed at reducing the contents of gramine- (**1**), tryptamine- (**2**) and tetrahydro-β-carboline-type (**3**) alkaloids usually associated with the toxic syndromes. This was extended to the tyramines (**4**), following identification of *N*-methyltyramine from *P. aquatica* using a rat atrial bioassay to isolate cardioactive components (Anderton et al., 1994).

A recent chemical assessment of *P. coerulescens*, as part of an agronomic investigation into its suitability for introduction into Australia, resulted in the isolation and identification of two oxindoles (**5** and **6**), and the observation of a major unidentified component (Anderton et al., 1998a; Anderton, Cockrum, Colegate, Edgar, & Flower, 1998). Unlike the oxindoles, this component behaved similarly to the usual *Phalaris*-derived tryptamine and tetrahydro-β-carboline alkaloids in the TLC screen (Anderton et al., 1998b). Subsequent isolation and purification of this alkaloid from *P. coerulescens* enabled its identification as a furano*bis*indole, a new class of alkaloid, which was given the trivial name, phalarine.

2. Results and discussion

The TLC and HPLC behavior of the laevorotatory phalarine indicated that it was less polar than the usual *Phalaris*-derived alkaloids (Anderton et al., 1998b). The colorless TLC spot for phalarine rapidly turns a yellow/brown color when exposed to UV light (254 nm). When sprayed with acidified anisaldehyde and gently warmed, the TLC spot turned pink/purple, similar to the reaction of the simple indole alkaloids. In order to establish whether phalarine is a true secondary metabolite or an artifact of the acid/base extraction and isolation procedure, fresh *P. coerulescens* was extracted with dichloromethane and the extract examined immediately by TLC. Spots characteristic of phalarine and the oxindoles (**5** and **6**) were clearly resolved, thereby indicating the natural occurrence of these alkaloids.

* Corresponding author.

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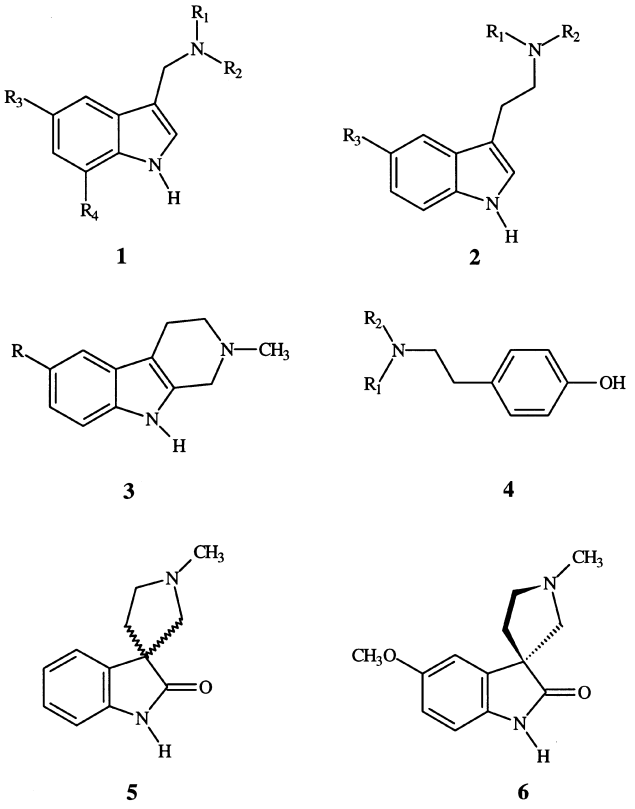
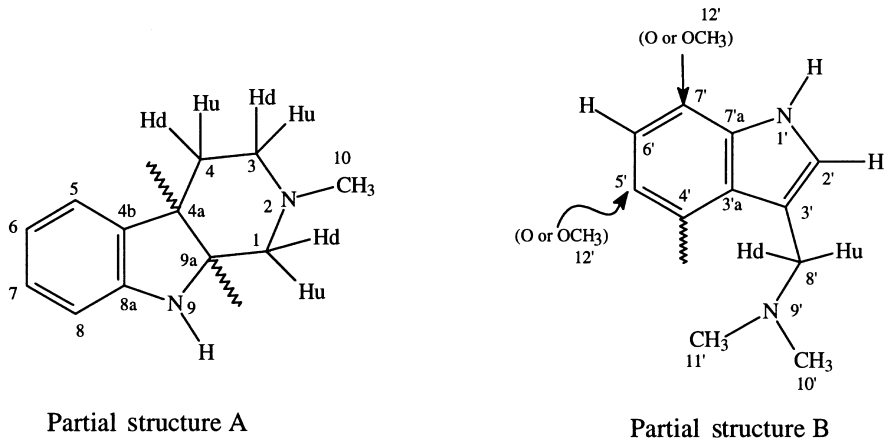
PII: S0031-9422(98)00725-0



Structure 1.

Phalarine was not detectable by GC mass spectrometry but a M_r of 404 was indicated by direct insertion probe (DIP) EI and by ESI or APCI ($[M+H]^+$, m/z 405). High resolution DIP-EI mass measurements suggested a molecular formula $C_{24}H_{28}N_4O_2$. ESI mass spectrometry of the acetylation (acetic anhydride-pyridine) or methylation (methyl iodide-potassium carbonate-acetone) products of phalarine indicated formation of a monoacetyl ($[M+H]^+$, m/z 447) or a monomethyl ($[M+H]^+$, m/z 419) derivative, respectively.

Extensive NMR investigation of phalarine and its monoacetyl derivative clearly established the presence of hexahydro-β-carboline (A) and oxygenated methoxygramine (B) partial structures. These structures were supported when the degree of fragmentation in the ESI mass spectrometry of phalarine and its derivatives was enhanced by increasing the cone voltage of the source. Product ions indicating losses of -NMe_2, -CH_2NMe_2, -OMe, -NMe, -$(CH_2)_2NMe$ and -$(CH_2)_2NMeCH_2$ were observed. APCI MS/MS on the $[M+H]^+$ for phalarine (m/z 405) confirmed the loss of $N(CH_3)_2$ to yield m/z 360, and subsequent APCI MS/MS on m/z 360 confirmed the



Structure 2.

losses of CH_3 (m/z 345) and $(CH_2)NCH_3$ (m/z 317). In addition to showing fragmentations similar to the ESI mass spectrometry, the DIP-EI mass spectrum of phalarine included ions at m/z 186 and 143, which are particularly supportive of the partial structure A in that the EI mass spectrum of 2-*N*-methyl-1,2,3,4-tetrahydro-β-carboline (**3**, R = H) shows a $[M]^+$ at m/z 186 and a base peak at m/z 143 corresponding to loss of $-CH_2NCH_3$ (Anderton et al., 1998b).

The proton-decoupled ^{13}C NMR spectrum of (−)-phalarine consisted of 22 sharp resonance signals and one low intensity, broadened resonance signal (δ 44.3), which was eventually assigned to a dimethylamine group. The total number of carbons detected thereby supported the proposed molecular formula based upon the high resolution mass measurement. A DEPT135 NMR experiment further defined the resonance signals as six methine, four methylene, four methyl (once again, the low intensity resonance signal for the dimethylamine group was very broad) and 10 quaternary carbons.

The assigned carbon chemical shifts and the related heteronuclear multiple quantum (HMQC) and heteronuclear multiple bond (HMBC) proton correlations for partial structures A and B are shown in Tables 1 and 2, respectively. For descriptive purposes in this report, the upfield chemical shift of a geminal proton pair is assigned to Hu and the downfield shift to Hd.

Observed proton–proton correlations (2-D-COSY) for phalarine (Table 3) also support the proposed partial structures. The relative configuration of the vicinal protons on carbons 3 and 4 of partial structure A is indicated by the magnitude of the coupling constants observed (Table 3). Whilst second order effects perturbed the multiplets, it was clearly evident that H3u and H4u each comprised one small and two large coupling constants, whilst their geminal partners H3d and H4d, each comprised one large and two small coupling constants. This requires H3u and H4u to be spatially opposed to each other in a diaxial relationship.

Placement of the proton at C6′ of partial structure B was indicated by the HMBC observations, whilst the chemical shift of C6′ (δ 88.7) is consistent with this carbon being flanked by oxygen-bearing carbons (Parmar et al., 1990; Colegate, Din, Ghisalberti, & Latiff, 1992). This then requires that C4′ forms the direct link with partial structure A as indicated by the HMBC observed between the proton resonance at δ 2.25 (H1u) and the carbon resonance at δ 113.4. Clearly, the nonmethylated oxygen substituent on partial structure B (C5′ or C7′) is involved in completing the fusion by bonding to C4a (δ 91.5) on partial structure A. Of these two possibilities, only bond formation leading to a furan-linked fusion of partial structures A and B is structurally feasible, thereby placing the methoxyl substituent at C7′, rather than C5′, resulting in structure **7** for phalarine. The absolute stereochemistry about carbons 4a and 9a remains undefined.

Table 1
Carbon chemical shifts with single and multiple bond proton correlations supporting partial structure A

Carbon	δ	HMQC	HMBC
1	65.4	2.25, d, 3.41, d	H3u, H3d, H10
3	50.6	2.13, ddd, 2.71, bd	H1u, H1d, H10, H4u, H4d
4	33.9	2.03, ddd, 2.62, bd	H3u, H3d
4a	91.5	–	H1d, H3d, H4d, H5
4b	133.5	–	H6, H8
5	122.6	7.30, d	H7
6	118.2	6.68,dd	H8
7	128.8	6.99, ddd	H5, H6
8	110.9	6.5, d	H6
8a	150.8	–	H5, H7
9a	72.1	–	H1d, H4d
10	46.7	2.35, 3H, s	H1u, H1d

Table 2
Carbon chemical shifts with single and multiple bond proton correlations supporting partial structure B

Carbon	δ	HMQC	HMBC
2′	125.4	7.02, d	H8u′, H8d′
3′	111.9	–	H2′, H8u′, H8d′, N1′-H
3′a	121.9	–	H2′, H8u′, H8d′, N1′-H
4′	113.4	–	H6′, H1u (part. Struct. A)
5′/7′	153.1	–	H6′
6′	88.7	6.32, s	–
7′/5′	146.3	–	H6′, H12′
7′a	124.0	–	H2′, H6′
8′	56.1	3.14, d, 3.95, d	H11′, H10′
11′,10′	44.3	2.28, 6H, s	H8u′, H8d′
12′	55.3	3.84, 3H, s	–

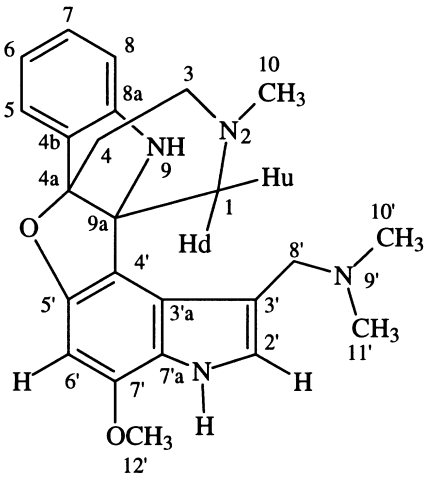


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Table 3
^{1}H chemical shifts and assignments, 2D-COSY NMR interactions and ^{1}H–^{1}H coupling constants for (−)-phalarine

δ	Multiplicity	Assignment	Proton–proton correlations and ^{1}H–^{1}H coupling constants
2.03	ddd	H4u	2.62 (H4d, J=14.5 Hz), 2.13 (H3u, J=ca. 13 Hz), 2.71 (H3d, weak correlation, J=ca. 4.8 Hz)
2.13	ddd	H3u	2.71 (H3d, J=10 Hz), 2.03 (H4u, J=ca. 13 Hz), 2.62 (H4d, J=ca. 2.2 Hz, correlation not observed)
2.25	d	H1u	3.41 (H1d, J=12.1 Hz)
2.28	s	H11′, H12′	–
2.35	s	H10	–
2.62	broad d	H4d	2.03 (H4u, J=14.5 Hz)
2.71	broad d	H3d	2.13 (H3u, J=10.0 Hz)), 2.03 (H4u), 3.41 (H1d, weak correlation)
3.14	d	H8′u	3.95 (H8′d, J=12.9 Hz)
3.41	d	H1d	2.25 (H1u, J=12.1 Hz), 2.71 (H3d, weak correlation)
3.84	s	H12′	–
3.95	d	H8′d	3.14 (H8′u, J=12.9 Hz), 7.02 (H2′)
6.32	s	H6′	–
6.50	d	H8	6.99 (H7, J=7.8 Hz)
6.68	dd	H6	6.99 (H7, J=7.4 Hz), 7.30 (H5, J=7.4 Hz)
6.99	ddd	H7	6.50 (H8, J=7.8 Hz), 6.68 (H6, J=7.4 Hz), 7.30 (H5, weak correlation)
7.02	d	H2′	3.95 (H8′d), 8.3 (N1′-H, J=2.3 Hz)
7.30	d	H5	6.68 (H6, J=7.4 Hz)
8.30	broad s	N1′-H	7.02 (H2′)
8.90	very broad s	N9-H	–

A 2-D-NOESY experiment led to the observation of a very weak nuclear Overhauser effect between the methoxyl protons (δ 3.84, H12′) and the indole nitrogen proton (δ 8.3, N1′-H). Although very weak, this nOe was also observed in the NOESY spectrum of the monoacetyl derivative of phalarine. This supports placement of the methoxyl group at C7′. Another nOe between the same methoxyl protons and the singlet at δ 6.32 indicated an *ortho*-relationship, thereby supporting the placement of the lone aromatic proton at C6′, as deduced from the HMBC experiments.

The nOes between geminal or vicinal protons were observed as expected for structure **7**. The protons H8′u and H8′d each displayed a clear interaction with the N9′-dimethyl signal at δ 2.28. Significantly, H8′d also displayed a strong interaction with H1d (δ 3.41), whilst H8′u interacted with H2′ (δ 7.02). Further nOes between H1d and the N9′-dimethyl protons confirmed the spatial proximity of H1d to the dimethylaminomethylene substituent at C3′. NOEs between H8 (δ 6.5) and the N9′-dimethyl protons, and between H5 (δ 7.30) and H4d (δ 2.62), confirmed the expected spatial proximity of these protons. Examination of 3-D models of structure **7** and measuring interatomic distances using a structure modeling computer program (Alchemy III, Tripos Associates), clearly indicated that the observed nOes are consistent with those that would be expected based upon spatial proximity.

Examination of the NMR data for the monoacetyl derivative of phalarine reinforced the structural deductions. Apart from the observation of appropriate ^{13}C and ^{1}H resonance signals for the acetyl substituent, and minor changes in the chemical shifts of some resonances, the major observation was a marked deshielding of protons in the spatial vicinity of the introduced carbonyl group. Thus, the C8′ geminal protons were deshielded to δ 4.75 and 5.3, the N9′ methyls were shifted to δ 3.45, H8 was deshielded to δ 7.2 and H1d was deshielded to δ 4.5. These observations support the expectation that it is N9 that is acetylated or methylated.

The representation of phalarine as structure **7** is consistent with the previous isolation of both 2-*N*-methyl-1,2,3,4-tetrahydro-β-carboline (**3**, R=H) and 5,7-dimethoxygramine (**1**, $R_1=R_2=CH_3$ and $R_3=R_4=OCH_3$) from *Phalaris* spp. (Gander, Marum, Marten, & Hovin, 1976; Mulvena, Picker, Ridley, & Slaytor, 1983). Whilst it can be postulated that a possible biosynthetic source of phalarine might involve oxidative coupling of the tetrahydro-β-carboline with the dimethoxygramine (or its 5-hydroxy precursor), only the 2-*N*-methyltetrahydro-β-carboline has so far been isolated from *P. coerulescens* (Anderton et al., 1998a, 1998b).

At present it is unknown whether phalarine is toxic to livestock. However, if *P. coerulescens* is to be considered for agronomic development and future widespread distribution, then it will be important to establish its toxicity.

3. Experimental

General experimental conditions, plant source and extraction were as previously reported (Anderton et al.,

1998a). A $CDCl_3$ soln of (−)-phalarine was used for the NMR experiments. ESIMS and APCIMS were recorded by infusing MeOH solns of phalarine into the source of a ThermoQuest-Finnigan LCQ ion trap mass spectrometer.

3.1. Isolation and identification of phalarine (7)

The alkaloidal fraction from *P. coerulescens* was subjected to repeated radial chromatography on silica gel plates using NH_4OH–MeOH–$CHCl_3$ (1:20:80) as eluent. Collected frns were monitored by TLC (NH_4OH–MeOH–$CHCl_3$ (1:10:40). Phalarine (R_f 0.8) was visualized by spraying with acidified anisaldehyde reagent, prepd by mixing anisaldehyde (2 ml), H_2SO_4 (2 ml), HOAc (0.4 ml) and EtOH (36 ml) and warming the sprayed plate gently. Under these conditions phalarine developed as a pink–purple spot similar to various indolylamine and tetrahydro-β-carboline alkaloids isolated from *Phalaris* spp. (Anderton et al., 1998b). HPLC of the purified phalarine showed a single peak (t_r ca. 60 mins, λ_{max} 274, 298 nm) when chromatographed using a cartridge packed with high purity silica with a C-18 bonded phase and end-capped (Merck Purospher RPe, 5 μm particle size, 150 × 4 mm) and eluted with a variable gradient of 0.1 vol% TFA in H_2O (solvent A) and 0.1 vol% TFA in MeOH (solvent B) (isocratic 2% B for 1 min; hyperbolic gradient 2–16% B over 28 min; hyperbolic gradient 16–36% B over 40 min; linear gradient 36–100% B over 11.5 min; isocratic at 100% B for 5 min and linear 100–2% B over 9.5 min) with a flow rate of 1 ml min^{-1}. (−)-Phalarine was thus obtained as an optically active, pale yellow oil (12 mg, 0.003% yield), $[\alpha]^{20}_D$ −92° (0.0075, CH_3OH). ^{13}C and ^1H NMR are recorded in Tabs. 1–3. EIMS (rel. intensity), m/z 404 ($[M]^+$, 50), 360 ($[M\text{-}N(CH_3)_2]^+$, 28), 359 ($[M\text{-}H\text{–}N(CH_3)_2]^+$, 40), 347 ($[M\text{–}CH_3N(CH_2)_2]^+$, 55), 303 ($m/z$ 347-$N(CH_3)_2$, 100), 217 (12), 186 (20), 158 (18), 143 (68). ESIMS and APCIMS, m/z 405 ($[M+H]^+$, 100%), 360 ($[M\text{-}N(CH_3)_2]^+$, 40). HREIMS $C_{24}H_{28}N_4O_2$ calcd 404.2212, found 404.2236, and $C_{22}H_{21}N_3O_2$ (M^+-H–$N(CH_3)_2$) calcd 359.1629, found 359.1634.

Acknowledgements

The HREIMS was recorded by I.Vit at the mass spectrometry facility of the CSIRO Division of Molecular Science, Clayton, Vict., Australia. The ESIMS of the monomethyl and monoacetyl derivatives of (−)-phalarine were recorded by G. Corrino at the mass spectrometry facility of the CSIRO Division of Wool Technology, Geelong, Vict., Australia.

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