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Pyrrolizidine alkaloids of the endemic Mexican genus *Pittocaulon* and assignment of stereoisomeric 1,2-saturated necine bases ☆

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Abstract

The endemic Mexican genus *Pittocaulon* (subtribe Tussilagininae, tribe Senecioneae, Asteraceae) belongs to a monophyletic group of genera distributed in Mexico and North America. The five *Pittocaulon* species represent shrubs with broom-like succulent branches. All species were found to contain pyrrolizidine alkaloids (PAs). With one exception (i.e., stems of *Pittocaulon velatum* are devoid of PAs) PAs were found in all plant organs with the highest levels (up to 0.3% of dry weight) in the flower heads. Three structural types of PAs were found: (1) macrocyclic otonecine esters, e.g. senkirkine and acetylpetasitenine; (2) macrocyclic retronecine esters, e.g. senecionine, only found in roots, and (3) monoesters of 1,2-saturated necines with angelic acid. For an unambiguous assignment of the different stereoisomeric 1,2-saturated necine bases a GC–MS method was established that allows the separation and identification of the four stereoisomers as their diacetyl or trimethylsilyl derivatives. All otonecine esters that generally do not form *N*-oxides and the 1,2-saturated PAs were exclusively found as free bases, while the 1,2-unsaturated 7-angeloylheliotridine occurring in *P. velatum* was found only as its *N*-oxide. In a comparative study the ^{1}H and ^{13}C NMR spectra of the four stereoisomeric necine bases were completely assigned by the use of DEPT-135, H,H-COSY, H,C-HSQC and H,H-NOESY experiments and by iterative analysis of the ^{1}H NMR spectra. Based on these methods the PA monoesters occurring in *Pittocaulon praecox* and *P. velatum* were assigned as 7-*O*-angeloyl ester respectively 9-*O*-angeloyl ester of dihydroxyheliotridane which could be identified for the first time as naturally occurring necine base. Unexpectedly, in the monoesters isolated from the three other *Pittocaulon* species dihydroxyheliotridane is replaced by the necine base turneforcidine with opposite configuration at C-1 and C-7. The species-specific and organ-typical PA profiles of the five *Pittocaulon* species are discussed in a biogenetic context.

Keywords: *Pittocaulon*; Pyrrolizidine alkaloids; Dihydroxyheliotridane; Turneforcidine; GC–MS analysis; Derivatization; NMR analysis

1. Introduction

Pittocaulon (ex *Senecio*) forms together with several related genera a monophyletic group within the subtribe Tussilagininae of the Senecioneae (Asteraceae). The genus *Pittocaulon* has been segregated from the huge cosmopolitan genus *Senecio*. This segregation is supported by means of classical methods (Robinson and Brettell, 1973) and by molecular phylogenetic techniques (Bain and Golden,

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2000). All five *Pittocaulon* species are endemic to Mexico with one also occurring in Guatemala. They are found in tropical dry habitats often on rocky outcrops or steep cliffs. They are shrubs or small trees with broom-like succulent branches with water-storing pith and bark (Olson, 2005). The branches bear small clusters of leaves during the rainy season and are leafless during the drought the rest of the year. The stems of *Pittocaulon praecox*, the most abundant species, are frequently infested by a scale insect, *Ceroplastes albolineatus* (Coccidae, Homoptera). We became interested in the chemistry of *Pittocaulon* when we observed that the phloem-feeding scale insect sequesters pyrrolizidine alkaloids (PAs) from its host plant (Marín-Loaiza et al., 2007).

The occurrence of PAs in most species of the genus *Senecio* s.l. is well established (Hartmann and Witte, 1995). Preliminary studies with *Pittocaulon* revealed the presence of two structural types of PAs: (i) 1,2-saturated and unsaturated PAs of the senecionine type (Hartmann and Witte, 1995) and (ii) simple angeloylmonoesters of 1,2-saturated and unsaturated necine bases. The co-occurrence of macrocyclic otonecine derivatives and simple 1,2-saturated monoesters is rarely observed among *Senecio* species. Preliminary observations indicated the presence of different stereoisomers of the 1,2-saturated necine base 1-hydroxymethyl-7-hydroxypyrrolizidine depending on the *Pittocaulon* species analyzed. Since there is a great inconsistency in the literature about the correct assignment of these stereoisomeric 1,2-saturated necines occurring in plants we included in our investigation the elaboration of a GC–MS method for a complete separation and unequivocal assignment of the four possible stereoisomers of 1-hydroxymethyl-7-hydroxypyrrolizidine as well as their structural identification and assignment by 1D and 2D NMR techniques.

2. Results

2.1. The PA profiles of the Pittocaulon species

The five Mexican *Pittocaulon* species were analyzed for the presence of PAs by GC–MS. The results are summarized in Table 1. The genus was found to contain a rich diversity of 17 different PAs. Two structural types were dominating in all species: (1) O^7- respectively O^9-monoesters of 1,2-saturated necine bases (Figs. 1b), and (2) macrocyclic diesters of the 1,2-unsaturated necine base otonecine (Fig. 1d). In two species, i.e. *Pittocaulon filare* and *P. velatum*, the 1,2-saturated monoesters are accompanied by mono esters of the 1,2-unsaturated necine base retronecine or heliotridine, respectively (Fig. 1b-1). The macrocyclic retronecine diester senecionine (**10**) and its *Z*/*E* isomer integerrimine (**11**) (Fig. 1c) which are structural analogs of the otonecine diesters senkirkine (**14**) and neosenkirkine (**15**) could be detected in the roots of three species. In *P. praecox*, senecionine and integerrimine were found together with their 1,2-saturated analogs platyphylline (**12**) and neoplatyphylline (**13**) (Fig. 1c). With one exception (stems of *P. velatum*) PAs could be detected in all analyzed plant organs but substantial qualitative and quantitative differences were observed between different organs. Flower heads displayed the highest PA concentrations reaching levels up to 0.3% (on a dry weight basis) in *Pittocaulon bombycophole*. This corroborates results from other *Senecio* species which also showed the highest PA levels in the inflorescences (Hartmann and Zimmer, 1986).

Whereas senecionine (**10**) and senkirkine (**14**) are frequently found in distant taxa within the tribe Senecioneae, the ligularidines (**17**, **18**) and petasitenines (**19**, **20**) so far are only found in taxa of the subtribe Tussilagininae, such as Asian and European *Petasites* and *Homogyne* species and the Asian *Farfugium* and *Ligularia* species (Hartmann and Witte, 1995). From the chemosystematic point of view this supports the classification of *Pittocaulon* as member of the Tussilagininae.

The co-occurrence of macrocyclic otonecine diesters and retronecine diesters is not surprising since the otonecine derivatives originate biogenetically from retronecine derivatives as it has been demonstrated for the conversion of senecionine into senkirkine (Toppel et al., 1987; Kelly et al., 1989). The occurrence of senecionine only in roots (Table 1) appears reasonable since in all *Senecio* species so far studied the roots are the exclusive site of senecionine biosynthesis (Hartmann et al., 1989; Hartmann and Ober, 2000). The various species-specific macrocyclic retronecine and otonecine derivatives are formed by peripheral modification of the basic backbone structure senecionine (Hartmann and Dierich, 1998; Pelser et al., 2005).

A particular feature of *Pittocaulon* is the quantitatively more or less balanced co-occurrence of macrocyclic PA diesters and PA monoesters. In the Senecioneae PA monoesters or open-chain diesters are rarely found together with macrocyclic PAs (Hartmann and Witte, 1995). Moreover, it is remarkable that the angeloylesters (**5**,**6**) of dihydroxyheliotridane (**4**) (Fig. 1a and b) that are found in two species (i.e., *P. praecox* and *P. velatum*) in the other three species are replaced by the respective esters (**7**,**8**) of turneforcidine (**2**) (Fig. 1a and b). In the course of the identification of the saturated necine bases an unequivocal assignment of the four possible stereoisomers (**1**–**4**) (Fig. 1a) was impossible with the tools so far available in literature. Therefore we established a GC–MS method allowing the separation and assignment of the four stereoisomers (see Section 2.2) and confirmed the structural assignment by comparative NMR (see Section 2.3). With these tools in hands an unequivocal assignment and identification of the stereoisomeric necines and their esters presented here was accomplished. The occurrence of dihydroxyheliotridane (**4**) as a plant constituent was new, until now it was only known as synthetic product (Robertson et al., 1996). Turneforcidine (**2**) has been frequently described as necine base of

Table 1
Organ-specific PA profiles and total PA concentrations in different organs of five *Pittocaulon* species established by GC–MS

	m/z $[M]^+$	RI(ZB1)	Pyrrolizidine alkaloids (relative abundance, %)														
			P. praecox				*P. bombycophole*			*P. filare*		*P. hintonii*			*P. velatum*		
			Ste[a]	Flo[a]	Lea[a]	Roo[a]	Ste[a]	Flo[a]	Roo[a]	Ste[a]	Roo[a]	Ste[a]	Flo[a]	Roo[a]	Ste[a]	Flo[a]	Roo[a]
7-Angeloylretronecine (**9**)	237	1809								43	16						
7-Angeloylheliotridine (**9a**)	237	1810														31[b]	(25)[c]
7-Angeloylturneforcidine (**7**)	239	1780					76	16	38	57	23	71	39	19			
9-Angeloylturneforcidine (**8**)	239	1806					8	6	6			3	2	1			
7-Angeloyldihydroxyheliotridane (**5**)	239	1810	33	66	78	tr									tr	65[b]	(25)[c]
9-Angeloyldihydroxyheliotridane (**6**)	239	1837		11	2												
Senecionine (**10**)	335	2284				40					tr						59
Integerrimine (**11**)	335	2342				16					tr						16
Platyphylline (**12**)	337	2335				4											
Neoplatyphylline (**13**)	337	2363				4											
Senkirkine (**14**)	365	2459	66	14		36	tr	6	8		10	1	1	12			tr
Neosenkirkine (**15**)	365	2534											1	3			
Petasitenine (**19**)	381	2447					tr						3	3			
PA-X (**X**)[d]	381	2542			12												
Acetylsenkirkine (**16**)	407	2632									tr	4	2	12			
Ligularidine (**17**)[e]	407	2708										tr	tr	1			
Neoligularidine (**18**)[e]	407	2709						1	tr		tr						
Acetylpetasitenine (**20**)	423	2632					16	70	48	tr	52	20	50	49			tr
mg/g dry wt:			0.13	0.97	1.42	0.46	2.41	3.04	0.52	0.16	0.25	1.12	2.89	0.99		0.32	0.16

[a] Ste: Stems; Flo: Flowers; Lea: Leaves; Roo: Roots.
[b] Angeloylesters **9** and **5** are not separated; they were determined after hydrolysis and quantification of the respective necine bases, see Table 2.
[c] Angeloylesters **9** and **5** are not separated; the sum of both compounds account for 25% rel. abundance.
[d] Unknown pyrrolizidine alkaloid.
[e] Elution sequence of the two *E*/*Z* - isomers not assured.

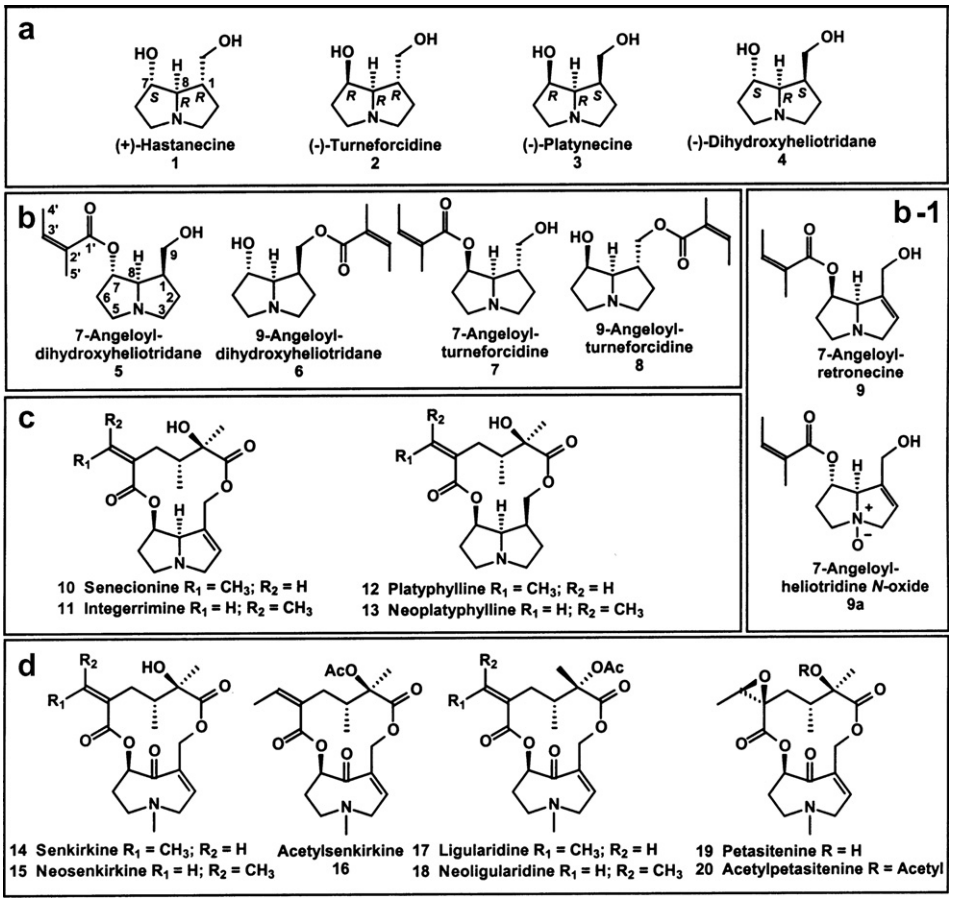

Fig. 1. Structures of the stereoisomeric 1,2-saturated necine bases (a) and PA esters identified from genus *Pittocaulon*.b: 1,2-saturated monoesters; b-1: 1,2-unsaturated monoesters; c: macrocyclic 1,2-unsaturated retronecine, respectively, 1,2-saturated platynecine diesters; d: 1,2-unsaturated macrocyclic otonecine diesters.

various PA monoesters and open-chain diesters as well as macrocyclic PAs of the monocrotaline type (Mattocks, 1986; Rizk, 1991; Hartmann and Witte, 1995). The third stereoisomeric necine base, platynecine (**3**), is the common necine base of almost all known 1,2-saturated macrocyclic PAs of the senecionine type like platyphylline (**12**) and neoplatyphylline (**13**) found in roots of *P. praecox* (Table 1). A biosynthetic relation between the macrocyclic PAs (Fig. 1c and d) and turneforcidine (Fig. 1a) appears reasonable since both have *R*-configuration at C-7. This applies for three of the *Pittocaulon* species but not for *P. praecox* and *P. velatum*. In both species 7*R*-configured macrocyclic PAs co-occur with the 7*S*-configurated dihydroxyheliotridane monoester. In *P. velatum* the saturated 7*S*-configurated monoester is accompanied by its 1,2-unsaturated analog 7-angeloylheliotridine (Tables 1 and 2). It appears unlikely that the 7*S*-configurated monoesters represent intermediates or off-branching side products in senecionine biosynthesis, unless one assumes an additional epimerization at C-7.

A particularity of *Pittocaulon* is the co-occurrence of PAs as free bases (1,2-saturated angeloylesters) and *N*-oxides (1,2-unsaturated angeloylesters, e.g. in *P. velatum*) (Fig. 1b-1; Tables 1 and 2). It is well documented that in *Senecio* species 1,2-unsaturated PAs are synthesized, maintained and stored exclusively as *N*-oxides (Toppel et al., 1987; Hartmann et al., 1989; Hartmann and Dierich, 1998). However, in this respect 1,2-saturated PAs occurring in *Senecio* have never been studied before. All *Pittocaulon* samples analyzed contain 1,2-saturated PAs exclusively as free base (tertiary PA); not even traces of *N*-oxides were detected. This is the first evidence from a *Senecio* species that 1,2-saturated PAs are solely present as free bases. The detailed analysis of the 7-angeloylesters from *P. velatum* revealed that they represent a mixture of 65% 7-angeloyldihydroxyheliotridane (**5**) and 31% 7-angeloylheliotridine *N*-oxide (**9a**) (Tables 1 and 2; Fig. 1b-1). Unfortunately the samples from *P. filare* (stems and roots), *P. praecox* (roots) and *P. velatum* (roots) containing 1,2-unsaturated PAs (Table 1) were only analyzed after reduction. Therefore it was not possible to decide whether the 1,2-unsaturated PAs were present as *N*-oxides or as free base. However the data from *P. velatum* flowers suggest that 1,2-unsaturated PAs found in the genus *Pittocaulon* may be present as *N*-oxides like in other *Senecio* species. 1,2-Saturated

Table 2
Pittocaulon velatum (flowers) contain two 7-angeloylesters that could not be separated by GC (DB1); 32% of the mixture is present as *N*-oxide

Parameter	M^+	RI	PAs (free base)	Total PAs (free base + *N*-oxide)[a]
7-Angeloyl esters	237/239	1810		
Relative abundance (%)			100	>95
mg/g dry wt			0.65	0.95
Present as *N*-oxide (%)			0	32
Hydrolysate of 7-angeloyl esters				
Dihydroxyheliotridane	157	1458		
Relative abundance (%)			100	65
Present as *N*-oxide (%)			0	0
Heliotridine	155	1446		
Relative abundance (%)			nd	31
Present as *N*-oxide (%)				97

Hydrolysis of the ester mixture yielded the necine bases dihydroxyheliotridane and heliotridine. Dihydroxyheliotridane is present exclusively as free base (tertiary PA), heliotridine exclusively as *N*-oxide.

[a] The ester fraction contained >5% of a PA tentatively identified as macrophylline (M^+ 239; RI 1861); the hydrolysate contained ca. 4% of the respective necine base most likely macronecine (M^+ 157; RI 1261). nd = not detected.

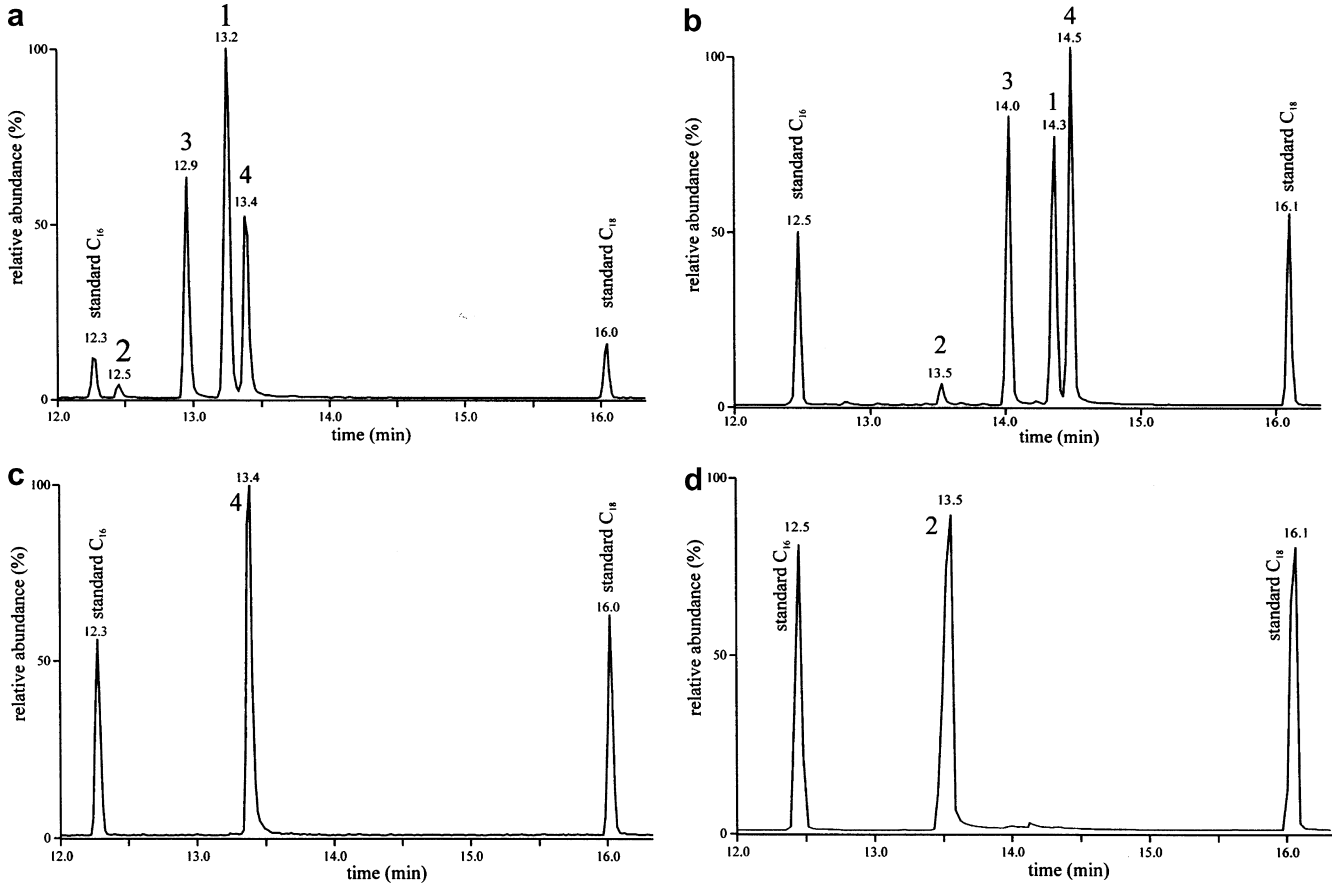

Fig. 2. GC-separation of the di-*O*-acetyl derivatives of the four stereoisomeric 1,2-saturated necine bases. a and b: Separations of standard mixtures; c: necine base obtained by hydrolysis of 7-angeloyldihydroxyheliotridane (**5**) from *P. praecox*; d: necine base obtained by hydrolysis of 7-angeloylturneforcidine (**7**) from *P. bombycophole*. a and c: separation of a ZB2 column; b and d: separation of a ZB1 column.

PAs occurring in other plant families are present either exclusively as tertiary PAs, i.e. Convolvulaceae (Jenett-Siems et al., 1998) or as mixtures of free bases and their *N*-oxides, i.e. Orchidaceae (Frölich et al., 2006). Otonecine derivatives like senkirkine due to structural reasons (*N*-methylation) do not form *N*-oxides (Toppel et al., 1987).

2.2. Separation of the 1,2-saturated necine bases and their esters by GC–MS

The necine bases **3** and **4** were prepared by hydrogenation of retronecine and heliotridine, respectively, using slightly modified procedures described by Donohoe et al.

(2002). Necine base **2** was obtained by hydrolysis of **7**, isolated from *P. bombycophole* and *Pittocaulon hintonii*. Necine **1** was a gift from S.E. Denmark. The identity of the four stereoismeric saturated necine bases were confirmed by GC–MS, optical rotation and NMR (see Section 2.3).

Simultaneous gas chromatographic separation of the four stereoisomeric saturated necine bases was achieved after *O*-trimethylsilylation (TMS-derivatives) or acetylations (acetyl derivatives). Disadvantage of the TMS-derivatives is a poor separation of hastanecine (ZB-1, RI = 1613) and platynecine (ZB-1, RI = 1615). Baseline separation was obtained with the acetyl derivatives using either a ZB-1 or a more polar ZB-5 column (Fig. 2).

After hydrolysis of the PA-monoesters this separation method was used for the assignment of the involved necine bases of the isolated 1,2-saturated PA monoesters from *P. praecox* (Fig. 2c) or the other *Pittocaulon* species (Fig. 2d).

The procedure can easily be adopted to identify and correctly assign the saturated necine bases **1**–**4** from any PA source. All reference compounds can be easily obtained by the catalytic hydrogenation starting from retronecine or heliotridine (see Section 4.4.). The identification of the necine bases is as well possible for sub-mg quantities from hydrolyzed PA mixtures since the most common unsaturated necine bases, i.e. retronecine and heliotridine are separated from the 1,2-saturated necine bases (see RI values in Section 4.5) and show different fragmentation patterns in EI-MS.

2.3. Identification of the 1,2-saturated necine bases and their monoesters by NMR spectroscopy

The ^{1}H (Tables 3 and 5) and ^{13}C NMR (Tables 4 and 6) spectra of the necine bases and their esters were completely assigned by the use of DEPT-135, H,H-COSY, H,C-HSQC and H,H-NOESY experiments. Moreover, the ^{1}H NMR spectra were analyzed by iteration on the full line shape (Weber and Thiele, 1998) to obtain a complete set of ^{1}H chemical shifts and H,H coupling constants. This was successful for all compounds with the exception of the protons H-2β, H-6α and H-6β of **7** which had strongly overlapping proton signals in the region $\delta = 2.12$–2.18 ppm. As a consequence, $J(6\alpha,6\beta)$ is not defined and the values of $J(6\alpha,7)$ and $J(6\beta,7)$ are interchangeable for this compound. The theoretical 400 MHz ^{1}H NMR spectra of the necine alcohols are reproduced in Fig. 3. They were obtained by iterating chemical shifts, coupling constants and linewidths. The good agreement between calculated and experimental spectra is indicated by *R* values between 0.8% and 2.0%.

Characteristic features in the ^{1}H NMR spectra are the very similar ($\Delta\delta = 0.01$ ppm) and the distinctly different ($\Delta\delta = 0.18$ ppm) chemical shifts of protons H-9a and H-9b in **3** and **4**, respectively, while the differences in **1** and **2** are small and identical ($\Delta\delta = 0.04$ ppm). Protons H-6α and H-6β have only small shift differences in **2** and **3** ($\Delta\delta = 0.01$ and 0.02 ppm, respectively) but large differences in **1** and **4** ($\Delta\delta = 0.33$ and 0.32 ppm, respectively). While **1** is characterized by its strong shielding of H-1 ($\delta_{H\text{-}1} = 1.94$ ppm), isomers **2**–**4** have $\delta_{H\text{-}1}$ values between 2.44 and 2.53 ppm. The large sum of the coupling constants involving H-7 ($\sum J_7 = 21.6$ Hz), typical for **4**, needs also mentioning although this value is decreased to 15.4 Hz in its ester **5** (see below). Finally, both **1** ($\delta_{H\text{-}2\alpha} = 1.53$ ppm) and **4** ($\delta_{H\text{-}2\beta} = 1.53$ ppm) feature a substantially higher shielding of one of their protons at C-2 than the other diastereomers: $\delta_{H\text{-}2\alpha} = 1.68$ and 1.74 ppm in **2** and **3**, respectively.

For the angeloyl esters H,C-HMBC spectra were recorded to secure the attachment of the acyl moieties to *O*-7 or *O*-9 from the crosspeaks between the carboxyl ^{13}C chemical shift and the ^{1}H chemical shift of H-7 and H-9, respectively. For all the necines studied, we found that both ^{1}H and ^{13}C chemical shifts, in particular those of the α-positions with respect to nitrogen (positions 3, 5 and 8), were not well reproducible when NMR measurements were repeated after storing compounds in $CDCl_3$ or in CD_3OD even for a few days. Chemical shift alterations of up to 2 ppm (^{13}C) and up to 0.7 ppm (^{1}H) were observed, which made many comparisons of spectra meaningless. Such behavior was also emphasized by S.E. Denmark (personal communication) and is most likely attributed to the easy protonation of the necine nitrogen atom. We found that reproducible NMR spectra of the necines were obtained when a few milligrams of these are dissolved in CD_3OD and two or three drops of ND_3 (25% in D_2O, Aldrich) are added to guarantee that the necines are present in solution as the free bases. Hence all spectra reported here were recorded under such conditions. Thus we can present a compatible set of ^{1}H and ^{13}C NMR data, including stereospecific proton assignments, for the stereoisomers dihydroxyheliotridane (**4**), hastanecine (**1**), platynecine (**3**), and turneforcidine (**2**) and the three monoesters **5**–**7**. Our ^{13}C NMR data of the necine bases show excellent agreement with the following literature values: **4** (Mulzer and Scharp, 1993), **1** (Fleet et al., 1991; Mulzer and Scharp, 1993; Denmark and Thorarensen, 1994), **3** (Fleet et al., 1991), **2** (Wee, 2001).

The two PA monoesters (**5**, **6**) isolated from *P. praecox* are both angelates as evidenced by their ^{1}H and ^{13}C NMR chemical shifts and EI-MS. The HMBC correlation between the angeloyl carboxyl carbon atom and the 9-protons showed that **6** is a 9-*O*-angeloyl derivative. This was deduced from the chemical shifts of the 9-protons ($\delta_H = 4.45$ and 4.22 ppm) whose signals are easily identified by their mutual geminal coupling constant of (−)11.2 Hz and their vicinal couplings with H-1 of 6.3 and 8.1 Hz, respectively. The H,H-NOEs (Table 5) and the ^{13}C chemical shifts of the necine part (Table 6) prove that **6** is 9-*O*-angeloyl-dihydroxyheliotridane: C-9 is deshielded by 1.9 ppm and C-1 shielded by 3.0 ppm relative to the values in **4**, all other ^{13}C chemical shift differences, $\Delta\delta_C$, between **6** and **4** being less than or equal to

Table 3
^{1}H NMR data ($CD_3OD + ND_3$) of the stereoisomeric 1,2-saturated necine bases

	Hastanecine (**1**)[a]	Turneforcidine (**2**)	Platynecine (**3**)	Dihydroxyheliotridane (**4**)
^{1}H NMR chemical shifts, δ_H				
1α	–	–	2.45	2.44
1β	1.94	2.53	–	–
2α	1.53	1.68	1.74	1.81
2β	1.97	2.10	1.98	1.53
3α	3.05	3.08	3.10	2.89
3β	2.53	2.58	2.79	2.65
5α	3.09	3.03	3.23	3.19
5β	2.70	2.70	2.86	2.59
6α	1.72	*2.00*	1.88	1.74
6β	2.05	*1.99*	1.90	2.06
7α	–	4.18	4.25	–
7β	4.09	–	–	4.12
8α	2.94	3.16	3.29	3.25
9a	3.62	3.57	3.94	3.85
9b	3.58	3.53	3.93	3.67
Coupling constants, J(H,H)				
1,2α	9.9	9.5	7.4	6.6
1,2β	6.6	7.2	11.9	10.5
1,8	7.5	7.1	8.0	7.9
1,9a	6.3	6.5	5.9	7.1
1,9b	6.9	7.1	5.1	7.7
2α,2β	−12.6	−12.3	−11.8	−12.4
2α,3α	6.8	6.8	7.0	6.0
2α,3β	10.3	10.2	2.6	3.1
2β,3α	2.9	2.9	9.8	9.9
2β,3β	5.9	6.0	9.0	7.5
3α,3β	−10.0	−9.6	−10.8	−11.3
5α,5β	−11.3	−9.8	−9.9	−10.5
5α,6α	5.9	7.3	8.3	7.2
5α,6β	9.1	2.9	1.2	3.7
5β,6α	4.4	10.8	11.9	9.8
5β,6β	7.0	6.3	6.3	6.1
6α,6β	−13.1	−12.1	−13.3	−12.2
6α,7	3.6	4.4	3.6	8.1
6β,7	5.3	2.6	1.3	6.4
7,8	2.6	4.6	3.2	7.1
9a,9b	−10.9	−10.5	−11.1	−11.1
$\sum J_7$	11.5	11.6	8.1	21.6
Stereochemically relevant NOEs				
	1β + 2β,7β	1β,2β	1α,2α	1α,2α
	2α,3α	1β,3β	1α,8α	1α,8α
	2α,9a+b	2α,3α	2α,3α	2α,3α
	2β,3β	2α,8α	2β,3β	2β,3β
	3β,5β	2α,9a+b	3β,5β	2β,7β
	5α,6α	2β,3β	5α,6α	2β,9a+b
	5β,6β	3β,5β	6α,7α	5α,6α
	6β,7β	7α,8α	7α,8α	5β,6β
	8α,9a+b	8α,9a+b		5β,7β
				6β,7β
				7β,9a+b

[a] To facilitate the comparison of the data of **1**–**4**, the configuration of C-8 is assumed to be always (*R*) and H-8 is pointing downwards (α). Hence the stereochemical descriptors in **1** are those for (+)-hastanecine.

0.4 ppm. All ^{1}H chemical shift differences between **6** and **4** are less than or equal to 0.15 ppm apart from those for H-9a and H-9b, which amount to 0.60 and 0.55 ppm, respectively. This ester has not been described so far. Similar reasons apply for the structural assignment of **5** to be 7-*O*-angeloyl-dihydroxyheliotridane. The HMBC spectrum exhibits a cross-peak between C=O and H-7 (δ_H = 5.26 ppm), H-7 is deshielded by 1.14 ppm relative to **4** (Table 5), and all $\Delta\delta_C$ values between **5** and **4** (Table 6) are less than or equal to 1.0 ppm with the exception of

Table 4
^{13}C NMR chemical shifts ($CD_3OD + ND_3$) of the stereoisomeric necine bases

C-	Hastanecine (**1**)	Turneforcidine (**2**)	Platynecine (**3**)	Dihydroxy-heliotridane (**4**)
1	47.7	40.8	45.0	44.9
2	30.9	32.6	28.9	28.6
3	55.3	56.2	56.5	55.1
5	53.0	52.8	54.8	53.9
6	33.5	37.5	37.3	35.8
7	77.9	72.0	73.2	72.0
8	76.6	73.7	72.6	73.2
9	65.3	65.6	61.6	63.2

C-7 (+3.7 ppm) and C-6 (−2.3 ppm). Apart from H-7 all ^1H chemical shift differences between **5** and **4** are less than or equal to 0.25 ppm. The H,H-NOEs (Table 5) confirm the aminoalcohol moiety of this ester to be dihydroxyheliotridane. Compound **5** shows excellent agreement of its ^1H and ^{13}C NMR data with those of an alkaloid which (Roeder and Liu, 1991) had isolated from *Senecio integrifolius* var. *fauiri* and to which they had attributed the structure of 7-*O*-angeloyl-turneforcidine. The criterion on which they based their structural decision was the half-height line width of the H-7 NMR signal (15 Hz), which equals $\sum J_7$, the sum of all coupling constants involving H-7, and which was close to the value reported for **2** (14.1 Hz) (Aasen et al., 1969).

The $\sum J_7$ rule had been used previously to infer the relative configurations of C-1, C-7, and C-8 of substituted PAs (Culvenor and Woods, 1965). However, it can only hold if the coupling constants do not change substantially upon esterification or other substitution of the necine alcohol, i.e. if the conformational equilibrium is not affected. The imponderabilities of this method have been emphasized (Culvenor et al., 1965; Logie et al., 1995). Our present $\sum J_7$ values for **5** (15.4 Hz, Table 5) and **4** (21.6 Hz, Table 3) again confirm the unreliability of the $\sum J_7$ rule. Nuclear Overhauser effects are a much safer method to derive the configurations of the pyrrolizidine alkaloids.

The PA monoester **7** isolated from *P. bombycophole* is 7-*O*-angeloylturneforcidine. Its chemical and NMR spectroscopic properties are different from the compound described by Roeder and Liu (1991). Again, the esterification of the 7-OH group follows from the HMBC correlation of the carboxyl carbon chemical shift with the one of H-7 ($\delta_H = 5.28$ ppm, Table 5). Proton H-7 is deshielded by 1.10 ppm relative to turneforcidine ($\delta_H = 4.18$ ppm, Table 3), all other proton chemical shifts being affected by 0.25 ppm or less. Among the ^{13}C chemical shifts of **7**,

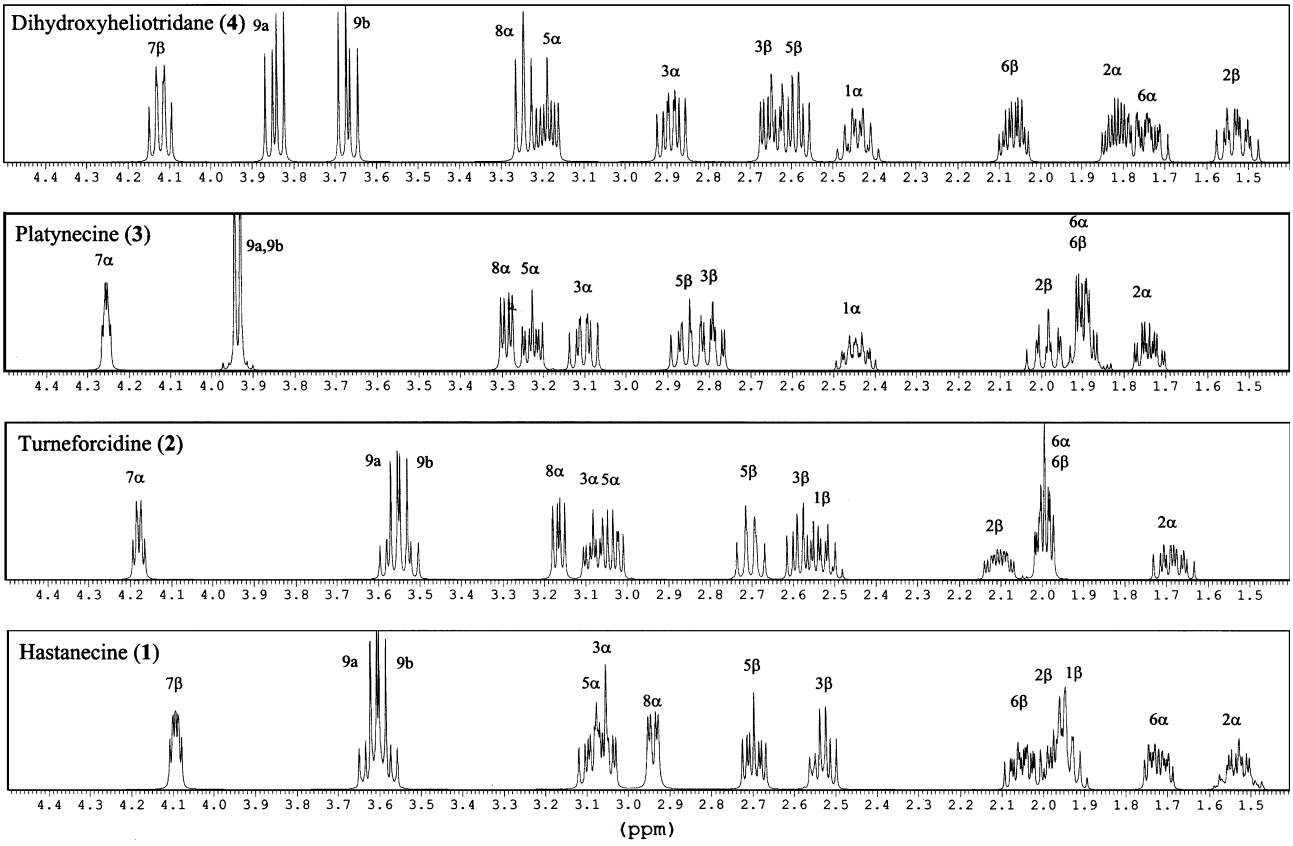

Fig. 3. Calculated 400 MHz ^1H NMR spectra of the 1,2-saturated necine alcohols hastanecine (**1**), turneforcidine (**2**), platynecine (**3**), and dihydroxyheliotridane (**4**) in CD_3OD/ND_3 solutions obtained by iterative fitting of the total line shape.

Table 5
^{1}H NMR data ($CD_3OD + ND_3$) of the isomeric necine angelates

H-	7-*O*-Angeloyl-dihydroxyheliotridane (**5**)	$\Delta\delta_H$	9-*O*-Angeloyl-dihydroxyheliotridane (**6**)	$\Delta\delta_H$	7-*O*-Angeloyl-turneforcidine (**7**)	$\Delta\delta_H$[a]
1α	2.49	0.05	2.59	0.15	–	–
1β	–	–	–	–	2.32	−0.21
2α	1.88	0.07	1.86	0.05	1.75	0.07
2β	1.64	0.11	1.62	0.09	ca 2.12	0.02
3α	2.98	0.09	2.90	0.01	3.12	0.04
3β	2.72	0.07	2.68	0.03	2.61	0.03
5α	3.17	−0.02	3.19	0.00	3.15	0.12
5β	2.83	0.24	2.61	0.02	2.70	0.00
6α	1.84	0.10	1.75	0.01	ca 2.18	0.18
6β	2.20	0.14	2.07	0.01	ca 2.12	0.13
7α	–	–	–	–	5.28	**1.10**
7β	5.26	**1.14**	4.11	−0.01	–	–
8α	3.50	0.25	3.25	0.00	3.41	0.25
9a	3.78	−0.07	4.45	**0.60**	3.54	−0.03
9b	3.63	−0.04	4.22	**0.55**	3.52	−0.01
3′	6.15		6.14		6.18	
4′	1.96		1.97		1.99	
5′	1.87		1.89		1.91	
Coupling constants, J (H,H)						
1,2α	6.8		6.5		9.1	
1,2β	9.4		10.7		6.6	
1,8	8.2		7.8		6.9	
1,9a	6.3		6.3		6.8	
1,9b	7.3		8.1		6.8	
2α,2β	−12.5		−12.4		−12.5	
2α,3α	6.4		5.9		6.7	
2α,3β	4.4		2.9		10.2	
2β,3α	8.6		10.0		3.1	
2β,3β	7.5		7.5		6.0	
3α,3β	−11.1		−11.4		−9.7	
5α,5β	−11.3		−10.5		−10.1	
5α,6α	7.3		7.1		7.6	
5α,6β	6.6		3.9		2.8	
5β,6α	5.9		9.6		10.8	
5β,6β	7.0		6.2		6.3	
6α,6β	−13.8		−12.3		?[b]	
6α,7	4.5		7.8		4.5[c]	
6β,7	6.5		6.4		*1.9*	
7,8	4.4		6.9		4.6	
9a,9b	−10.9		−11.2		−10.7	
3′,4′	7.3		7.3		7.3	
3′,5′	1.5		1.5		1.5	
4′,5′	1.6		1.6		1.6	
$\sum J_7$	15.4		21.1		11.0	
Stereochemically relevant NOEs						
	1α,2α		1α,2α		1β,2β	
	1α,3α		1α,8α		1β,3β	
	1α,8α		2α,3α		2α,3α	
	2α,3α		2β,3β		2α,9a+b	
	2β,3β		2β,7β		2β,3β	
	5α,6α		3β,5β		3β,5β	
	5β,6β		5α,6α		5α,6α	
	6β,7β		5β,6β		6α,7α	
	7β,9a+b		5β,7β		7α,8α	
			6β,7β		8α,9a+b	

[a] δ_H(necine ester)–δ_H(necine alcohol).
[b] Undetermined.
[c] The values of J(6α,7) and J(6β,7) may be interchanged.

$\delta_{C\text{-}7}$ is increased by 3.5 ppm and $\delta_{C\text{-}6}$ and $\delta_{C\text{-}8}$ are decreased by 1.7 and 1.8 ppm, respectively, relative to **2** as expected by an esterification of 7-OH. All other ^{13}C shifts are affected by 0.9 ppm or less. Nuclear Overhauser effects

Table 6
^{13}C NMR chemical shifts ($CD_3OD + ND_3$) of the isomeric necine angelates

C-	7-*O*-Angeloyl-dihydroxyheliotridane (**5**)	$\Delta\delta_C$	9-*O*-Angeloyl-dihydroxyheliotridane (**6**)	$\Delta\delta_C$	7-*O*-Angeloyl-turneforcidine (**7**)	$\Delta\delta_C$
1	44.9	0.0	41.9	**−3.0**	41.7	0.9
2	29.6	1.0	28.9	0.3	32.4	−0.2
3	54.8	−0.3	54.9	−0.2	56.1	−0.1
5	54.0	0.1	54.0	0.1	53.0	0.2
6	33.5	**−2.3**	36.1	0.3	35.8	**−1.7**
7	75.7	**3.7**	72.3	0.3	75.5	**3.5**
8	72.7	−0.5	72.8	−0.4	71.9	**−1.8**
9	62.8	−0.4	65.1	**1.9**	65.2	−0.4
1′	169.3		169.6		168.5	
2′	128.9		129.0		128.8	
3′	139.5		139.3		140.2	
4′	16.1		16.1		16.1	
5′	20.7		20.8		20.9	

δ_C(necine ester)–δ_C(necine alcohol).

(Table 5) confirmed the turneforcidine configuration of the necine base.

3. Conclusion

A specific feature of the PA profiles found in the genus *Pittocaulon* is the dominating side-by-side occurrence of simple angeloylmonoesters of 1,2-saturated necine bases and 1,2-unsaturated macrocyclic otonecine esters. The two types of PAs differ in their biological activities. The 1,2-unsaturated PAs are generally pro-toxins that are bioactivated by cytochrome P450 enzymes of the xenobiotic detoxification systems of vertebrates (Fu et al., 2004) and insects (Frei et al., 1992) causing fatal cytotoxic and mutagenic effects. On the other hand, 1,2-saturated PAs are not bioactivated and their effects on herbivores is still not well understood. They represent the only PA type present in alkaloid-producing orchids (Frölich et al., 2006) and most PA containing *Ipomoea* species (Convolvulaceae) (Jenett-Siems et al., 1998, 2005). They have been reported to be insect deterrents (Reina et al., 1997) and they are sequestered by insects that utilize plant PAs for their own protection (Hartmann et al., 2005a,b; Marín-Loaiza et al., 2007). The latter reference concerns sequestration of *Pittocaulon* PAs by a scale insect. The chemoecological function of the 1,2-saturated PAs needs further clarification.

From the chemical point of view the presence of the angeloyl esters of the necine base dihydroxyheliotridane in two *Pittocaulon* species and their complete replacement by the stereoisomeric necine base turneforcidine in the three remaining species is noteworthy. The unambiguous assignment of the correct configuration of the respective 1,2-saturated necine bases was achieved by a detailed GC–MS analysis and confirmation of the structures by NMR. This method can easily be applied to confirm the correct configuration of a 1,2-saturated necine base in an unknown alkaloids sample.

4. Experimental

4.1. Plant materials

P. praecox (Cav.) Rob. and Brett. was collected within the Campus of the Universidad Nacional Autónoma de México (UNAM) (2277 m), México D.F, México. Flowers, roots and stems were collected during spring (March 2004), leaves at fall (October, 2004). Flowers, roots and stems of *P. bombycophole* (Bullock) H. Rob. & Brett., *P. velatum* (Greenm.) H. Rob. & Brett., and *P. hintonii* H. Rob. & Brett. were collected in March 2005. Roots and stems of *P. filare* (McVaugh) H. Rob. & Brett. were collected in March 2005.

P. velatum and *P. hintonii*, were collected in the state of Michoacán, México along the road from Ciudad Hidalgo to Zitácuaro (2235 m) and Municipio of Coalcomán (Rancho la Parota) (1230 m), respectively. *P. filare* was collected about 5 km south of the intersection to Los Asmoles on the non-toll highway Colima-Manzaillo (464 m) in the state of Colima, México. *P. bombycophole* was collected along the road between Buenavista and Coaxcaclán, in the state of Guerrero, México at 1728 m. The botanical identification of the plants was done by Dr. Mark Olson (Institute of Biology, UNAM). Voucher Specimens are deposited at the Herbarium of the Institute of Biology, UNAM (MEXU). Plant materials were air-dried, grounded and stored dry until alkaloid extraction.

4.2. GC–EI-MS, routine GC and polarimetry

Configuration I. A Hewlett–Packard 5890A gas chromatograph equipped with a 30 m × 0.32 mm (f_t 0.25 μm) analytical column (ZB-1, Phenomenex, Aschaffenburg, Germany) was used. The capillary column was directly coupled to a TSQ 700 mass spectrometer (Finnigan, Bremen, Germany). The conditions applied were: Injector and transfer line were set at 250 °C; the temperature program used was: 100 °C (3 min)–6 °C/min–310 °C (3 min).

The injection volume was 1 μl. The split ratio was 1:20, the carrier gas flow was 1.6 ml min^{-1} He, and the mass spectra were recorded at 70 eV.

Configuration II. A Hewlett–Packard 6890N gas chromatograph equipped with a 30 m × 0.32 mm (f_t 0.25 μm) analytical column (DB-5, Agilent J&W, Waldbronn, Germany) was used. The capillary column was directly coupled to a GC Mate II mass spectrometer (Jeol, Tokyo, Japan). The conditions applied were: Injector and transfer line were set at 250 and 250 °C, respectively; the temperature program used was: 100 °C (3 min)–6 °C/min–310 °C (3 min). The injection volume was 1 μl. The split ratio was 1:5 to 1:20, the carrier gas flow was 1.5 ml min^{-1} He, and the mass spectra were recorded at 70 eV.

Routine gas chromatography (GC) was performed as described applying a 15 m × 0.25 mm (f_t 0.25 μm) fused-silica column (DB1, J&W Scientific) (Witte et al., 1993; Hartmann et al., 2004). Quantitative analyzes were performed via the FID signals with heliotrine or monocrotaline as internal standards.

Where possible, the optical activity of the isolated or synthesized compounds was determined with a Propol Polarimeter (Dr. Kernchen, Seelze-Letter, Germany) at 25–27 °C. The measurement was not applied to compounds that showed <95% purity (based on GC and NMR measurements).

4.3. NMR spectroscopy

^1H and ^{13}C NMR spectra were obtained on a Bruker DRX-400 spectrometer at 400.1 and 100.6 MHz, respectively, or on a Bruker Avance II-600 spectrometer at 600.1 and 150.9 MHz, respectively. The solvent was CD_3OD (ca. 0.65 ml) to which 2–3 drops of ND_3 (25% solution in D_2O, Aldrich) had been added. ^1H chemical shifts were referenced to internal tetramethylsilane (TMS, $\delta_H = 0.00$ ppm), ^{13}C chemical shifts to the solvent signal ($\delta_C = 49.0$ ppm). The quantity of the compounds dissolved ranged from 1.5 to 20 mg depending on their availablility. One-dimensional ^1H and ^{13}C spectra, DEPT-135 ^{13}C spectra and two-dimensional H,H-COSY, H,H-NOESY, H,C-HSQC and H,C-HMBC spectra were with standard Bruker pulse programs. The mixing delay in the NOESY experiments was 2.0 s. The one-dimensional ^1H NMR spectra were analyzed by iterative fitting of the full lineshape using Bruker's WIN-DAISY program version 4.05 (1998).

4.4. Preparation or origin of saturated necine bases

The starting compound retronecine was obtained by hydrolysis of monocrotaline in saturated aqueous BaOH at 100 °C for 2 h. (−)-Platynecine was prepared by catalytic hydrogenation of retronecine using a slightly modified procedure recently described by Donohoe et al. (2002). A solution of 23 mg retronecine in 4 ml anhydrous THF containing 1 mg rhodium on activated charcoal (Fluka) was stirred under atmospheric pressure of H_2 for 16 h in the dark. The catalyst was removed by centrifugation (1 min) and the solvent was evaporated under a stream of N_2. The residue was dissolved in 2 ml MeOH then filtrated and the solvent removed again under a stream of N_2. The oily residue (yield 20 mg) was crystallized from acetone at −18 °C. The purity was checked with GC and GC–MS analysis after derivatization with MSTFA. The resulting GC–MS chromatograms revealed two peaks with almost identical mass spectra. The main compound (92%, based on peak area) was as expected the (−)-platynecine derivative, the other compound (8%) the corresponding diasteromere at C-1 (−)-turneforcidine. The proportion of (−)-turneforcidine could be reduced to <3% when monocrotaline was directly hydrogenated with H_2 followed by a basic hydrolysis of the resulting 1,2-dihydromonocrotaline.

(−)-Dihydroxyheliotridane was prepared from heliotridine using the same method as described above for platynecine. The purity was checked with GC and GC–MS analysis after derivatization with MSTFA. The resulting GC–MS chromatograms revealed again two peaks with almost identical mass spectrua. The main compound (93%, based on peak area) was the expected (−)-dihydroxyheliotridane derivative, the other compound (7%) the corresponding diasteromere at C-1 (+)-hastanecine. The appearance of the corresponding minor diasteromere did not interfere with NMR measurements. In fact it showed to be helpful in establishing the GC-method to separate all four naturally occurring saturated necine bases. (−)-Dihydroxyheliotridane ($[\alpha]_D^{589}$ −15.7° (MeOH)) was also obtained in pure form after hydrolysis of naturally occurring 7-angeloyldihydroxyheliotridane.

(−)-Turneforcidine $[\alpha]_D^{589}$ +4.3° (MeOH) was obtained by hydrolysis of 7-angeloylturneforcidine isolated from *P. bombycophole* and *P. hintonii*.

(+)-Hastanecine $[\alpha]_D^{589}$ −10° (MeOH) was kindly supplied as a gift by Dr. S. E. Denmark.

4.5. Separation and characterization of the 1,2-saturated necine bases by GC/MS

Trimethylsilyl derivatives. The compounds (0.1–0.2 mg) were dissolved in 100 μl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) at 75 °C for 30 min and then directly analyzed by GC–MS.

7,9-Di-*O*-trimethylsilyl-turneforcidine. RI(ZB1) 1572, RI(DB5) 1581; GC–EIMS, *m/z* (rel. int.): 301 (3, $[M]^+$), 286 (6), 211 (15), 198 (2), 185 (65), 170 (2), 147 (3), 143 (6), 122 (3), 82 (100), 73 (16), 55 (2), 45 (2).

7,9-Di-*O*-trimethylsilyl-hastanecine. RI(ZB1) 1613, RI(DB5) 1626; GC–EIMS, *m/z* (rel. int.): 301 (2, $[M]^+$), 286 (4), 211 (17), 198 (2), 185 (62), 170 (1), 147 (3), 122 (2), 82 (100), 73 (13), 55 (1), 45 (2).

7,9-Di-*O*-trimethylsilyl-platynecine. RI(ZB1) 1615, RI(DB5) 1627; GC–EIMS, *m/z* (rel. int.): 301 (2, $[M]^+$), 286 (7), 211 (15), 198 (2), 185 (73), 170 (2), 147 (3), 143 (1), 122 (4), 82 (100), 73 (16), 55 (2), 45 (2).

7,9-Di-*O*-trimethylsilyl-dihydroxyheliotridane. RI(ZB1) 1628, RI(DB5) 1641; GC–EIMS, *m/z* (rel. int.): 301 (2, $[M]^+$), 286 (4), 211 (14), 198 (2), 185 (60), 170 (2), 147 (3), 143 (1), 122 (3), 82 (100), 73 (16), 55 (2), 45 (2).

1,2-Unsaturated necine bases (for comparison)

7,9-Di-*O*-trimethylsilyl-retronecine. RI(ZB1) 1602, RI(DB5) 1617; GC–EIMS, *m/z* (rel. int.): 299 (5, $[M]^+$), 284 (1), 210 (2), 208 (3), 183 (40), 168 (5), 140 (4), 120 (9), 116 (16), 106 (8), 103 (26), 94 (40), 93 (84), 80 (17), 75 (23), 73 (100), 67 (9), 59 (12), 53 (7), 45 (18).

7,9-Di-*O*-trimethylsilyl-heliotridine. RI(ZB1) 1637, RI(DB5) 1651; GC–EIMS, *m/z* (rel. int.): 299 (4, $[M]^+$), 284 (2), 210 (2), 208 (2), 183 (36), 168 (5), 140 (4), 120 (8), 116 (15), 106 (11), 103 (25), 94 (25), 93 (83), 80 (17), 75 (24), 73 (100), 67 (9), 59 (11), 53 (7), 45 (17).

Acetylated derivatives. The compounds (0.5–1 mg) were dissolved in pyridine/Ac_2O (200 μl, 1:1 v/v) at 75 °C for 2 h and then directly analyzed by GC–MS.

7,9-Di-*O*-acetyl-turneforcidine. RI(ZB1) 1609, RI(DB5) 1659; GC–EIMS, *m/z* (rel. int.): 241 (3, $[M]^+$), 198 (4), 182 (18), 181 (36), 168 (4), 155 (7), 138 (48), 121 (18), 108 (7), 95 (46), 82 (100), 68 (4), 55 (9).

7,9-Di-*O*-acetyl-platynecine. RI(ZB1) 1636, RI(DB5) 1687; GC–EIMS, *m/z* (rel. int.): 241 (2, $[M]^+$), 198 (3), 182 (20), 181 (44), 168 (3), 155 (6), 138 (43), 121 (17), 108 (6), 95 (42), 82 (100), 68 (4), 55 (9).

7,9-Di-*O*-acetyl-hastanecine. RI(ZB1) 1652, RI(DB5) 1704; GC–EIMS, *m/z* (rel. int.): 241 (2, $[M]^+$), 198 (3), 182 (18), 181 (32), 168 (3), 155 (6), 138 (42), 121 (26), 108 (8), 95 (40), 82 (100), 68 (4), 55 (10).

7,9-Di-*O*-acetyl-dihydroxyheliotridane. RI(ZB1) 1659, RI(DB5) 1712; GC–EIMS, *m/z* (rel. int.): 241 (1, $[M]^+$), 198 (2), 182 (21), 181 (41), 168 (2), 155 (4), 138 (38), 121 (32), 108 (9), 95 (35), 82 (100), 68 (5), 55 (11).

1,2-Unsaturated necine bases (for comparison).

7,9-Di-*O*-acetyl-retronecine. RI(ZB1) 1625, RI(DB5) 1712; GC–EIMS, *m/z* (rel. int.): 239 (18, $[M]^+$), 197 (36), 180 (32), 179 (34), 168 (3), 153 (10), 136 (36), 120 (24), 119 (30), 118 (7), 106 (8), 101 (12), 94 (37), 93 (100), 80 (18), 67 (4), 53 (7).

7,9-Di-*O*-acetyl-heliotridine. RI(ZB1) 1652, RI(DB5) 1705; GC–EIMS, *m/z* (rel. int.): 239 (9, $[M]^+$), 197 (24), 180 (25), 179 (23), 168 (3), 153 (11), 136 (35), 120 (21), 119 (49), 118 (6), 106 (13), 101 (13), 94 (38), 93 (100), 80 (21), 67 (6), 53 (10).

4.6. Isolation of the 1,2-saturated PA monoesters

Isolation and identification of monoesters **5** and **6** from *P. praecox:* Powdered plant materials (leaves, flowers) were defatted by Soxhlet extraction with petroleum ether (40 °C–60 °C) and then the alkaloids Soxhlet-extracted with MeOH. The solvent was evaporated under reduced pressure and the residue dissolved in 1 M HCl, filtered and extracted with CH_2Cl_2. Zinc dust was added to the acidic aqueous solution for 3 h to reduce all PA *N*-oxides. The mixture was filtered, made basic to pH 11 and applied to an Extrelut (Merck) column. The alkaloid fraction was eluted with CH_2Cl_2 (l ml/g Extrelut). The solvent was evaporated and the residue directly applied to GC–MS analysis or HPLC.

Semi-preparative HPLC. Samples of 0.5 ml were applied to a RP-18 Column (Nucleosil 120-7, 250 mm long, 25 mm i.d.; Macherey & Nagel). Separation was achieved isocratically by using CH_3CN/TFA (25:75 v/v.), pH 2, at a flow rate of 9 ml min^{-1}; detection: absorbance at 210 nm. Compound **5**, RT = 13.7 min; compound **6**, RT = 15.7 min.

Monoester **7** was isolated from *P. bombyphocole* and *P. hintonii* using the same procedure as applied for the purification and separation of compounds **5** and **6** (HPLC, RT = 15.1 min).

4.7. Identification of 1,2-saturated PA monoesters

7-Angeloyl-dihydroxyheliotridane (**5**). $[\alpha]_D^{589}$ 7.5° (MeOH). RI(ZB1) 1811, RI(DB5) 1898; GC–EIMS, *m/z* (rel. int.): 239 (1, $[M]^+$), 221 (2), 156 (41), 139 (80), 138 (27), 108 (14), 106 (11), 96 (8), 95 (14), 82 (100), 68 (5), 55 (28), 53 (7).

9-Angeloyl-dihydroxyheliotridane (**6**). $[\alpha]_D^{589}$ −8.2° (MeOH). RI(ZB1) 1834, RI(DB5) 1911; GC–EIMS, *m/z* (rel. int.): 239 (10, $[M]^+$), 221 (17), 195 (3), 140 (11), 122 (13), 119 (6), 106 (4), 96 (41), 95 (100), 82 (90), 67 (3), 55 (23).

7-Angeloyl-turneforcidine (**7**). $[\alpha]_D^{589}$ +26.6° (MeOH). RI(ZB1) 1781, RI(DB5) 1845; GC–EIMS, *m/z* (rel. int.): 239 (4, $[M]^+$), 156 (60), 139 (100), 138 (17), 114 (3), 113 (20), 108 (7), 82 (60), 68 (2), 55 (9), 53 (2).

9-Angeloyl-turneforcidine (**8**). RI(ZB1) 1806, RI(DB5) 1869; GC–EIMS, *m/z* (rel. int.): 239 ($[M]^+$, 14), 221(23), 195 (3), 156 (15), 140 (15), 139 (24), 122 (21), 82 (100), 96 (49), 95 (99), 55 (20).

4.8. Identification of other PAs

Widely distributed and well characterized PAs like 7-angeloylretronecine (**9**), senecionine (**10**), integerrimine (**11**), platyphylline (**12**), neoplatyphylline (**13**), senkirkine (**14**) and neosenkirkine (**15**) were identified by their RIs, molecular ions and mass fragmentation patterns in comparison to reference compound and our comprehensive MS data base of PAs (Witte et al., 1993). Their GC–MS data are not shown. Some less abundant PAs were also identified by their GC–MS characteristics in comparison to reference compounds and the data base. Their GC–MS data are given below:

Petasitenine (**19**) RI(ZB1) 2442. GC–EIMS, *m/z* (rel. int.): 381 ($[M^+]$, 4), 353 (5), 294 (6), 266 (33), 250 (17), 168 (88), 152 (37), 151 (100), 124 (36), 123 (68), 122 (52), 110 (61), 108 (35), 96 (41), 70 (40).

Acetylsenkirkine (**16**) RI(ZB1) 2641. GC–EIMS, *m/z* (rel. int.): 407 ($[M^+]$, 1), 320 (2), 303 (8), 266 (6), 249 (11), 221 (5), 166 (25), 153 (48), 122 (30), 110 (48), 107 (26), 83 (24), 82 (29), 81 (24), 53 (29), 43 (100).

Ligularidine/neoligularidine (**17/18**). The *Z*/*E* isomers are separated by GC but the assignment is still uncertain; the MS fragmentation patterns were identical. RI(DB1) 2720 and 2709. GC–EIMS, *m*/*z* (rel. int.): 407 ([M^+], 2), 320 (7), 303 (30), 249 (98), 221 (16), 199 (29), 166 (34), 154 (33), 153 (100), 122 (47), 110 (70), 108 (17), 107 (52), 100 (28), 96 (29), 94 (27), 83 (34), 82 (39), 81 (38).

Acetylpetasitenine (**20**) RI(ZB1) 2632. GC–EIMS, *m*/*z* (rel. int.): 423 ([M^+], 5), 336 (79), 319 (43), 304 (44), 296 (13), 281 (11), 264 (20), 250 (61), 238 (22), 168 (61), 154 (34), 150 (86), 141 (42), 94 (37), 83 (35), 81 (34), 123 (46), 122 (92), 110 (79), 69 (86), 55 (36), 44 (70), 43 (100).

PA-X (otonecine type PA) RI(ZB1) 2542. GC–EIMS, *m*/*z* (rel. int.): 381 ([M]$^+$, 11), 182 (28), 168 (24), 153 (100), 144 (31), 109 (24), 100 (34), 83 (42), 82 (31), 58 (36), 57 (27).

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