Further Meroterpenes Produced by *Penicillium* sp., an Endophyte Obtained from *Melia azedarach*[#]

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Z. Naturforsch. **58c,** 663–669 (2003); received January 22/March 11, 2003

From the methanol extract of *Penicillium* sp. cultivated on sterilized rice for three weeks we isolated three new meroterpenes preaustinoid A1, A2 and B1. The fungus was isolated from the root bark of *Melia azedarach* after surface sterilization. The structures of these compounds were identified by intensive spectroscopic studies.

Key words: Melia azedarach, *Penicillium* sp., Meroterpenes

Introduction

The meroterpenes form a class of complex metabolites derived from a mixed terpenoid-polyketide biosynthetic pathway (Cornforth, 1968). These compounds have been isolated from a wide range of fungal species and marine organisms. The mixed terpenoid-polyketide route was confirmed for andibenin (**1**), by stable isotope labeling studies (Holker and Simpson, 1978). Subsequently, a large number of metabolites with a wide range of apparent structural diversity were shown to be biosynthetically closely related to andibenin (**1**), as exemplified by those compounds related with austin (**2**) (Simpson *et al*., 1997). These substances are all formed *via* a common intermediate that arises by alkylation of a polyketide intermediate, 3,5-dimethylorsellinate, with the terpenoid precursor farnesyl diphosphate (Simpson, 1998).

Only limited studies have been possible on the later stages of the biosynthetic pathways leading to these compounds. However, recent work on *Penicillium* sp associated with the Meliaceae plant *Melia azedarach*, has shown that it produces previously unknown compounds, called preaustinoids A (**3**) and B (**4**) (Santos and Rodrigues-Fo., 2002). These fungal metabolites had been postulated previously as intermediates of the biosynthetic pathway to austin (**2**) (Simpson *et al*., 1997).

Continuing our studies on the secondary metabolites produced by fungi associated with *Melia azedarach*, we now report the production, isolation and identification of three new meroterpenes, named preaustinoid A1 (**5**), A2 (**6**) and B1 (**7**), by *Penicillium* sp.

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin Elmer 241 polarimeter. UV spectra were obtained in CH_2Cl_2 solution on a Hewlett Packard 8452-A spectrophotometer, and IR spectra were measured with a Bomen MB-102 spectrophotometer in KBr pellets. HREIMS measurements were carried out on a VG-Autospec spectrometer, and electronic impact was used as ionization mode. Low-resolution APCIMS data were acquired in positive ion mode, using a MICROMASS QUATTRO-LC instrument equipped with an ESI/APCI "Z-spray" ion source. ^1H and ^{13}C NMR experiments were recorded on a BRUKER DRX-400 spectrometer with deutero chloroform (CDCl3) as the solvent and TMS as the internal standard.

Plant material

Melia azedarach roots were collected in São Carlos, Brazil, on the campus of the Federal University of São Carlos. A voucher specimen has been deposited in the Herbarium of the Department of Botanic at the University.

[#] This is part of the PhD thesis of RMGS.

0939–5075/2003/0900–0663 $ 06.00

Isolation of the microorganism

The general procedures adopted followed the methodology described by Petrini *et al.*, 1992. Immediately after collection, the root bark was separated mechanically from the wood and washed with water followed by ethanol and then sterilized with 11% aqueous sodium hypochloride for 1 min. The material was then deposited on a Petri dish containing PDA medium (potato-dextrose-agar) and incubated in the dark at 25 °C for one week. *Penicillium* sp was isolated by replication and grew as a bluish colored culture. The fungus was identified and deposited (LaBioMi024) at the Laboratório de Bioquímica Micromolecular – LaBioMi – of the Departamento de Química at Universidade Federal de São Carlos, São Carlos, Brazil.

Rice culture of Penicillium sp. *and isolation of the meroterpenes*

Fifty Erlenmeyer flasks (500 ml) containing about 90 g of rice (*"Uncle Ben's"*) and 75 ml of distilled water per flask were autoclaved twice at 121 °C for 40 min. A small disc of the PDA medium from the Petri dish containing mycelium of *Penicillium* sp. was transferred under sterile conditions to 47 of the 50 Erlenmeyer flasks containing sterilized rice. Three flasks were kept for control purposes. After 20 days of growth, the water that had accumulated in the flasks was separated by filtration from the fungal biomass. About 150 ml of methanol was added to each flask and allowed to stand for 5 h, after which it was filtrated by gravity. The methanol was evaporated under reduced pressure, producing a yellowish residue (6 g), which was subjected to a low-pressure silica gel CC eluted with a hexane to methanol gradient (hexane, hexane:dichloromethane 90:10, 70:30, 50:50, 30:70, dichloromethane, dichloromethane: methanol 99:1 until methanol 100%). The medium polarity fractions eluted with dichloromethane 1% methanol were reiteratively chromatographed on silica gel columns and the meroterpenes preaustinoid A1, A2 and B1 were finally purified by precipitation from methanol as a white powder.

Preaustinoid A1 (**5**)

White amorphous powder; m.p. 240.5–241.4 °C; $[\alpha]_D^{25}$ −25.55 (*c* 2.7, $CHCl_3$); UV λ_{max}/nm (CH_3OH): 210, 308(s); IR ν_{max} KBr cm^{-1} 3375, 1742, 1730, 1705, 1689; ^1H NMR (400 MHz, $CDCl_3$, standard $SiMe_4$): Table I; ^{13}C NMR (100 MHz, $CDCl_3$, standard $SiMe_4$) data obtained by pendant (Homer and Perry, 1995), HSQC and HMBC: Table I; APCIMS (daugther ions, 15 eV): *m/z* 461 (5) $[M+H]^+$, 429 (18), 411 (28), 393 (100), 369 (21), 351 (28), 323 (33), 273 (42), 235 (90), 167 (79); HREIMS *m/z* 460.24610. Calc. for $C_{26}H_{36}O_7$: 460.246103.

Preaustinoid A2 (**6**)

White amorphous powder; m.p. 243.9–246.0 °C; $[\alpha]_D^{25}$ −51.56 (*c* 7.7, $CHCl_3$); UV λ_{max}/nm (CH_3OH): 230(s); ν_{max} KBr cm^{-1} 3422, 1745, 1730, 1705, 1674, 1630; ^1H NMR (400 MHz, $CDCl_3$, standard $SiMe_4$): Table I; ^{13}C NMR (100 MHz, $CDCl_3$, standard $SiMe_4$) data obtained by pendant (Homer and Perry, 1995), HSQC and HMBC: Table I; APCIMS (daugther ions, 15 eV): *m/z* 459 (10) $[M+H]^+$, 441 (44), 423 (22), 409 (50), 391 (18), 349 (100); HREIMS *m/z* 458.2257. Calc. for $C_{26}H_{34}O_7$: 458.230453.

Preaustinoid B1 (**7**)

White amorphous powder; m.p. 46.4–48.0 °C; $[\alpha]_D^{25}$ −35.7 (*c* 2.7, $CHCl_3$); UV λ_{max}/nm (CH_3OH): 210, 222(s), 304(s); IR ν_{max} KBr cm^{-1}: 3405, 1758, 1739, 1706, 1622; ^1H NMR (400 MHz, $CDCl_3$, standard $SiMe_4$): Table I; ^{13}C NMR (100 MHz, $CDCl_3$, standard $SiMe_4$) data obtained by pendant (Homer and Perry, 1995), HSQC and HMBC: Table I; APCIMS (daugther ions, 15 eV): *m/z* 445 (21) $[M+H]^+$, 413 (37), 385 (10), 371 (10), 353 (100), 251 (12); HREIMS *m/z* 444.25119. Calc. for $C_{26}H_{36}O_6$: 444.251189.

Results and Discussion

The fungus *Penicillium* sp., which is under intense chemical investigation in our laboratory due to its intimate association with the plant *Melia azedarach*, produces several compounds in complex mixtures, which we assume, based on ^1H NMR spectra, to belong the meroterpene class of natural product. Most of these compounds are difficult to detect in an HPLC separation system

since they have no chromophores in their molecular structure. Our efforts to purify these complex mixtures using classical chromatography methods resulted in the identification of three previously nondescribed meroterpenes (**5**, **6** and **7**), which were co-produced with **3** and **4** (Fig. 1).

The molecular formula ($C_{26}H_{36}O_7$) for compound **5** was deduced based on HREIMS and APCI/MS which detected $M^{+\cdot}$ at *m/z* 460.24610 (calc. 460.24610) and $[M+H]^+$ at *m/z* 461, respectively. The IR spectrum of **5** showed a strong absorption at 3375 (hydroxyl group), 1742, 1730, 1705 and 1689 cm^{-1} (four carbonyl groups).

A comparison of the NMR data of compound **5** with those of preaustinoid A (**3**) indicated a close similarity between them. The preaustinoid A (**3**) has a 2-hydroxy-2-methyl-1,3-dioxo partial structure (ring D) which probably derived from the pentaketide 3,5-dimethylorsellinate after its alkylation by farnesyl diphosphate to form the meroterpene skeleton. Compound **5** has a methyl group (δ 1.37), which is correlated in the HMBC spectrum with a carbinolic carbon (δ 70.0, C-5′, ^2J) and two carbonyl carbons (δ 203.3, C-4′, ^3J; δ 207.1, C-6′, ^3J) (Fig. 2). These data and the comparison with the ^{13}C chemical shift for the carbons in the C and D rings of preaustinoid A confirmed the presence of a 2-hydroxy-2-methyl-1,3-dioxo partial structure in compound **5**.

The ^1H NMR data of **5** (Table I) indicated the presence of six tertiary methyl groups at δ 1.02, 1.29, 1.36, 1.36, 1.45 and 1.49, which correspond, respectively, to hydrogens, 3H-13, 3H-12, 3H-14, 3H-10′, 3H-15 e 3H-9′. Two of these methyl groups (δ 1.36, 3H-14 and 1.45, 3H-15) are geminal, according the HSQC and HMBC spectra, since they show correlation with the same quaternary carbon (δ 85.4, C-4, ^2J) and with each other methyl carbon (δ 30.6, C-15, ^3J and 27.0, C-14, ^3J). This time the methyl hydrogens at C-14 and C-15 do not shows HMBC with a ketone carbon signal (C-3) like observed for the meroterpenes **3** and **4**. Instead, the ^{13}C NMR spectrum of **5** contains a peak at δ 174.2, which was ascribed to C-3, whose chemical shift indicated it to be part of an ester or a lactone. These data, in conjunction with the

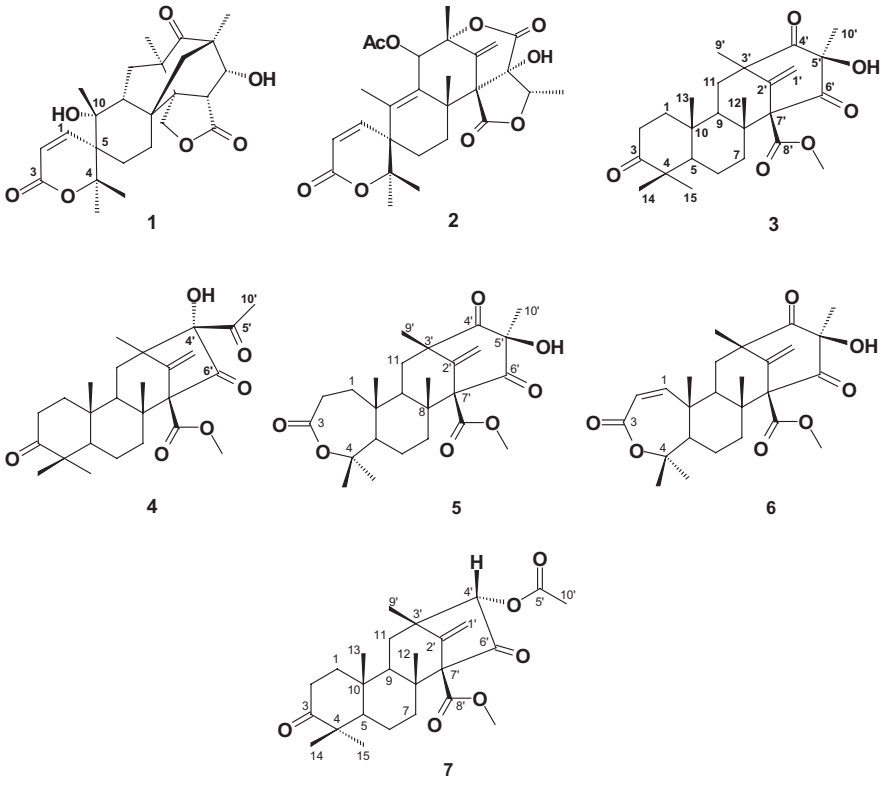

Fig. 1. Structures of meroterpenes produced by *Penicillium* sp. **1**: andibenin, **2**: austin, **3**: preaustinoid A, **4**: preaustinoid B, **5**: preaustinoid A1, **6**: preaustinoid A2, and **7**: preaustinoid B1.

Fig. 2. HMBC correlations detected for **5**, **6**, and **7**.

presence of a complex four spin system in its ^{1}H NMR spectrum (δ 1.12, H-1α; δ 1.70, H-1β; δ 2.41, H-2α; and δ 2.49, H-2β) and with the MS and IR data, suggested that the ring A of the meroterpene **5** is ε-lactone probably formed by a Bayer-Villiger like oxidation of the cyclohexanone in preaustinoid A (**3**). Therefore, this apparently new meroterpene was named preaustinoid A1.

Finally, the relative configurations at the stereocenters of **5** were proposed through an analysis of the following 1D-NOE data (NOE-DIFF and gNOESY). Irradiation of the signal at δ 4.90 (H-1′a) in the ^{1}H NMR spectrum produced an enhancement of the signals at δ 5.43 (H-1′b), 3.73 (OCH_3) and 1.29 (3H-12), while irradiation of the signal at δ 5.43 (H-1′b) enhanced the singlet at δ 1.49 (3H-9′). These and other irradiations are presented in Fig. 3.

The analysis of the molecular formula of **6** ($C_{26}H_{34}O_7$), deduced from the mass spectra obtained by HREIMS ($M^{+\cdot}$ found at 458.2257; calc. 458.2304) and APCI/MS ($[M+H]^+$ at *m/z* 459) indicated that it contains 10 double bond equivalent (DBE), one more than calculated for compound **5**. In addition to the MS data, its IR (3422, 1745, 1730, 1705 and 1674 cm^{-1}) and NMR (Table I) spectra indicated that **5** and **6** are two closely related meroterpenes, with identical B, C and D rings. The NMR spectrum of **6** (Table I) contains a pair of doublet corresponding to *cis*-olefinic hydrogens at δ 5.81 (*d*, *J* = 12 Hz) and 6.04 (*d*, *J* = 12 Hz) for H-2 and H-1, respectively, in ring A. The correlation of H-1 (^3J) and H-2 (^2J) with a carbonyl carbon at δ 167.6 in the HMBC spectrum clearly indicated that the ring A of the meroterpene **6** is formed by an α,β-unsaturated-ε-lactone. These data and other long-range correlations are summarized in Fig. 2. Differential NOE studies (gNOESY and NOEDiff) contributed to the final elucidation of this structure, in which irradiation of H-12 (δ 1.33) produced an enhancement of the H-1′a, H-11β, H-7β, H-13 and the methoxyl signal, respectively, at δ 4.92, 1.75, 2.24, 1.18 and 3.74. In addition, the methyl group CH_3-10′ (δ 1.37) showed a NOE with H-9 (δ 0.65), indicating that compound **6** includes a hydroxyl group at the *exo*-face. These and other results are presented in Fig. 3. This meroterpene was named preaustinoid A2, since it is structurally related to the two previous preaustinoids **3** and **5**.

The APCI mass spectrum of **7** exhibited an abundant ion $[M+H]^+$ detected at *m/z* 445. Its

Table I. NMR spectroscopic data for meroterpenes **5–7** ($CDCl_3$, 400 for ^1H and 100 MHz for ^{13}C).

	5		**6**		**7**	
Pos.	H	C	H	C	H	C
1α	1.12 *ddd* (14; 14; 5)	39.7 *t*	6.04 *d* (12)	157.3 *d*		38.6 *t*[a]
					1.45 *dd* (13; 13)	
1β	1.70 *m*				1.83 *ddd* (13; 3; 3)	
2α	2.41 *ddd* (14; 5; 5)	32.1 *t*	5.81 *d* (12)	122.5 *d*	2.44 *m*	33.7 *t*
2β	2.49 *ddd* (14; 14; 5)				2.49 *m*	
3	–	174.2 *s*	–	167.6 *s*	–	217.1 *s*
4	–	85.4 *s*	–	85.6 *s*	–	47.3 *s*
5α	1.63 *m*	51.7 *d*	1.79 *dd* (13; 3)	56.0 *d*	1.38 *m*	54.3 *d*
6α	1.47 *m*	23.4 *t*	1.49 *m*	23.3 *t*	1.57 *ddd* (10; 7; 4)	19.8 *t*
6β	1.70 *m*		1.70 *m*		1.56 *m*	
7α	1.89 *ddd* (13; 13; 4)	31.8 *t*	1.87 *ddd* (13; 13; 3)	31.5 *t*	1.77 *ddd* (13; 13; 4)	31.5 *t*
7β	2.23 *ddd* (13; 4; 4)		2.24 *ddd* (13; 3; 3)		2.03 *ddd* (13; 4; 4)	
8	–	47.7 *s*	–	47.3 *s*	–	46.9 *s*
9α	0.56 *dd* (13; 3)	52.6 *d*	0.65 *dd* (13; 3)	49.4 *d*	1.30 *dd* (13; 4)	50.8 *d*
10	–	39.7 *s*	–	43.2 *s*	–	37.2 *s*
11α	1.93 *dd* (13; 3)	39.6 *t*	2.0 *dd* (13; 3)	39.9 *t*	1.44 *m*	31.9 *t*
11β	1.65 *dd* (13; 13)		1.75 *dd* (13; 13)		1.83 *dd* (13; 13)	
12	1.29 *s*	16.3 *q*	1.33 *s*	16.6 *q*	1.21 *s*	16.5 *q*
13	1.02 *s*	18.6 *q*	1.18 *s*	14.7 *q*	0.91 *s*	16.2 *q*
14	1.36 *s*	27.0 *q*	1.39 *s*	32.5 *q*	1.02 *s*	20.8 *q*
15	1.45 *s*	30.6 *q*	1.42 *s*	27.1 *q*	1.07 *s*	26.5 *q*
1′a	4.9 *s*	112.9 *t*	4.92 *s*	113.1 *t*	4.87 *s*	107.5 *t*
1′b	5.43 *s*		5.45 *s*		5.15 *s*	
2′	–	145.5 *s*	–	145.3 *s*	–	148.1 *s*
3′	–	51.1 *s*	–	51.1 *s*	–	45.7 *s*
4′	–	207.3 *s*	–	207.9 *s*	5.12 *s*	80.5 *d*
5′	–	80.6 *s*	–	80.6 *s*	–	169.8 *s*
6′	–	204.0 *s*	–	203.4 *s*	–	206.6 *s*
7′	–	72.5 *s*	–	72.2 *s*	–	70.7 *s*
8′	–	168.5 *s*	–	168.2 *s*	–	168.0 *s*
9′	1.49 *s*	22.1 *q*	1.52 *s*	21.9 *q*	1.23 *s*	20.6 *q*
10′	1.36 *s*	14.9 *q*	1.37 *s*	14.9 *q*	2.21 *s*	21.2 *q*
OCH_3	3.73 *s*	53.0 *q*	3.74 *s*	52.6 *q*	3.65 *s*	52.0 *q*
OH	3.26 *s*	–	3.28 *s*	–	–	–

[a] ^{13}C Multiplicity was obtained through analysis of pendant and HSQC spectra.

molecular formula ($C_{29}H_{32}O_{11}$) was obtained from HREIMS data (calc. 444.2512; experimental 444.2508). The IR spectrum showed absorptions at 1758 (acetyl group), 1739 (carbomethoxyl group), 1706 (ketone) and 1622 cm^{-1} (vinyl group).

The ^1H NMR spectrum of **7** (Table I), analyzed with the aid of 2D NMR data (COSY, HMBC), showed the presence of a partial structure containing A, B and C rings with a ketone group located at C-3, identical with those seen in the preaustinoids A (**3**) and B (**4**) (Santos and Rodrigues-Fo., 2002). This was established by the ^1H-^{13}C three bonds correlation between two methyl groups [δ 1.02 (3H-14) and 1.07 (3H-15)] with a carbonyl carbon (δ 217.1) in the HMBC spectrum and the ^{13}C chemical shift comparison of the carbons of rings B and C in **7** and **4**. Other ^1H-^{13}C long-range correlations corroborated with this purpose and they are summarized in Fig. 2.

The ^{13}C NMR spectrum of **7** showed signals for one additional ketone carbonyl at δ 206.6 (C-6′), one carboxymethyl ester at δ 168.0 (C-8′), and an exocyclic carbon-carbon double bond (δ 107.5 and 148.1) located at C-2′. According to the NMR data [^1H: δ 2.21 (*s*, H-10′), ^{13}C: δ 169.8 (C-5′) and 21.2 (C-10′)] and the IR spectrum (1758 cm^{-1}), the meroterpene **7** contains an acetyl group. The hydrogen signal at δ 5.12 (*s*), a high-deshielded carbinolic proton (H-4′) showed correlation with a ketone carbon at δ 206.6 (C-6′), with an acetoxy carbonyl at δ 169.8 (C-5′) and with a methylene (CH_2) signal at δ 31.9 (C-11). These data are consistent with the presence of an α-acetoxy-cyclopentanone as the ring D of this meroterpene.

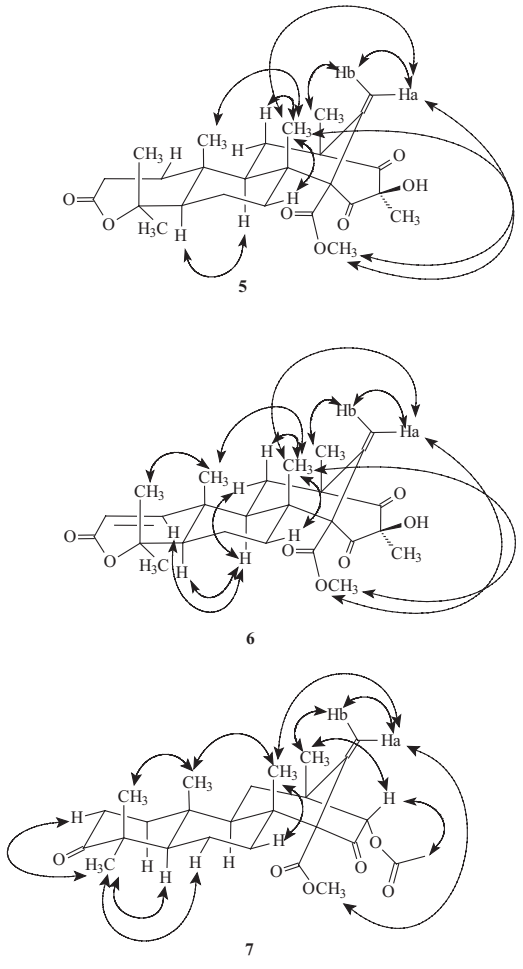

Fig. 3. Spatial correlations suggested for **5**, **6**, and **7** based on NOE difference spectra. Ha and Hb correspond, respectively, to vinyllic hydrogens H-1′a and H-1′b.

The final assignment of the structure of **7** was achieved from NOE studies (gNOESY), which are presented in Fig. 3. Irradiation of H-1′a resulted in enhancement of the H-1′b, a vinyl hydrogen at δ 4.87, and also the methyl group 3H-12 and the methyl ester group, respectively, at δ 1.21 and 3.65. Finally, the irradiation of the signal at δ 5.12 (H-4′) showed an enhancement in the methyl group at δ 1.23 (H-9′) suggesting that the acetoxy group is located at the *endo*-face in the [3:2:1]-bicyclo system while the carbinolic hydrogen H-4′ is located at the *exo*-face. Thus, the structure of this meroterpene, named preaustinoid B1, was identified as **7** and is apparently a new natural product. We numbered the carbons in the acetyl group as C-5′ (carbonyl) and C-10′ (methyl) for convenience only. Although chemically the acetyl can be formed from the methyl-ketone **4**, it is still to be proved whether these two carbons are part of the 3,5-dimethylorsellinate precursor or result from a normal natural acetylation reaction.

The host plant, from which the meroterpenoid producer fungus was obtained, is a producer of limonoids, which are degraded triterpenoids. During the limonoid biosynthesis, the triterpene precursors suffer many reactions equivalent to Bayer-Villiger oxidation and structural rearrangements (Waterman and Grundon, 1983). The striking similarity between the modifications found in the limonoids and in the meroterpenoids strongly suggests that the enzyme activities responsible for the modifications in these compounds may be the result of a plasmid exchange encoding limonoid or meroterpene biosynthetic genes between these two organism. This is now under investigation in our laboratory.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) for financial support and research fellowships.

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