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Degradation of the neolignan, burchellin in the hemolymph of the bloodsucking insect *Rhodnius prolixus*

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Abstract

The neolignan, burchellin, a natural compound that reduces urine excretion in larvae of the bloodsucking bug, *Rhodnius prolixus*, a vector of Chagas' disease, is rapidly degraded in the hemolymph of the insect. The main product that accumulates in this tissue has been shown to be piperonyl alcohol. Other catabolites have been identified by GC–MS analysis.

Keyword: Rhodnius prolixus; Neolignan; Burchellin; Biodegradation; Hemolymph

1. Introduction

Lignans and neolignans are phenylpropanoids widely distributed among Angiosperms [1]. This class of metabolites has a large array of biological properties, such as antineoplasic [2], antifungal [3], antibacterial [4] and antihypertensive activities [5]. On insects, lignoids have been described as potent feeding deterrents [6,7] and growth inhibitors [8]. They also mimic juvenile hormones [9], and act synergistically with a variety of insecticides, increasing their toxicity [10]. Biological activities of lignans associated with plant–insect chemical interaction have been recently reviewed [11]. As part of a general study aimed towards reduction of the transmission of Chagas' disease [12], a major endemic disease in Latin America [13], we have recently investigated the effects of lignoids on the hematophagous bug, *Rhodnius prolixus*, one of the vectors of the protozoan *Trypanosoma cruzi*, the causative agent of the illness.

In previous papers, we have established that lignoids exerted antifeeding and antimoulting activities against *R. prolixus* larvae [14–16]. We also showed that some neolignans inhibit *T. cruzi* infection of the triatomine insect vector [17,18]. Among them, burchellin (**1**) reduced considerably the excretion of the insect [15,18,19]. The effect of this treatment was partially reversed by simultaneous serotonin therapy [19]. Since urine excretion in *R. prolixus* is a major step to complete meal digestion – and hence insect ecdysis – inhibition of this process may constitute an efficient way for insect control.

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Table 1
GC–MS analysis of the hemolymph of *R. prolixus* fed on blood containing 100 μg ml^{-1} burchellin [a]

Peaks 30 min	Peaks 1 h	RT (min)	MS	Compounds
a_1	a_2	3.6	93 [b]-78-75-66-65-63-52	Aniline
b_1	b_2	6.3	135-108-91-83-77-74-70-63-51 [b]	Piperonyl alcohol
–	c_2	13.8	154-125-111-96-84-70-69-56 [b]	7-Methyl-4-decene
–	d_2	15.8	206-177-154-135-119-104-91-84-77-63-51	n.i.
–	e_2	16.7	234-205-192-151-135-117-104-91-84-77 [b]-70-63-56	n.i.
–	f_2	17.3	232-203-171-150-135 [b]-119-109-91-84-77-69-63-51	n.i.
–	g_2	17.6	248-213-198-158-151-135-99-91-84-77 [b]-65-51	n.i.
–	h_2	18.7	186 [b]-154-144-125-117-116-109-104-98-91-89-82-70-63-56	7-Methyl-4-decene MeOH adduct
c_1	i_2	20.3	300 [b]-285-267-239-194-181-152-135-127-115-103-91-77-69-51	Compound **6**
d_1	j_2	21.3	340 [b]-205-162-135-115-103-91-77-65-63-53-51	Burchellin
–	k_2	23.0	338-263-219-209-189-178-160-149-139-128-115-101-91-77-65 [b]-63-53	Dehydroburchellin

[a] Hemolymph was collected 30 min and 1 h after the bloodmeal, respectively.
[b] Base peaks; n.i. = not identified.

However, the structural complexity of burchellin **1** discouraged large-scale synthesis of this compound, for its use in Chagas' disease prophylaxis. It thus became relevant to look for the minimal structural arrangement responsible for the activity. A starting hypothesis was made that the bioactivity could be associated to some metabolites, resulting from burchellin **1** degradation, which should accumulate in the hemolymph. Accordingly, we investigated herein the degradation of burchellin **1** in the hemolymph of *R. prolixus*.

2. Experimental

2.1. Burchellin preparation and feeding procedure

Burchellin (**1**), afforded by Otto R. Gottlieb, was isolated from *Aniba burchelli* Kostern (Lauraceae) wood in the Amazon region [20]. The compound was dissolved in acetone and added to a 1:4 EtOH–saline solution (stock). Two groups of 20 to 30 carefully-staged fourth-instar larvae were starved for a 25–30 days, weighed and then allowed to feed on citrated human blood, using a feeder membrane device described previously [21]. An aliquot of the stock solution was added to the bloodmeal in order to obtain a final concentration of 100 μg of **1** per ml of blood. Control group was fed on blood added with EtOH–saline only, and the test group allowed to feed on blood containing **1**. At the end of the meal, the insects were weighed again to establish the amount of ingested blood. Only fully gorged insects were considered. Both groups of insects were maintained at 28 °C during all the experiment.

2.2. Analysis of the hemolymph

The hemolymph was collected by cutting the hind legs, using a microsyringe, 30 min (1st experiment) and 1 h (2nd experiment) after the end of the bloodmeal. The hemolymph was submitted to GC–MS analysis on a HP-5 capillary column (i.d.: 0.32 mm; length: 30 m; film thickness: 0.25 μm), using a Hewlett-Packard instrument, model 6890, equipped with a FID detector (t^o 310 °C), operating in the program mode from 170 to 300 °C, temperature increase of 2.5 °C min^{-1}, maintaining the upper limit for 20 min; mobile phase: H_2 (flow: 3.6 ml min^{-1}); software control: HPCHEM/1. Hemolymph (1 μl) was injected in the split mode (1:20); the temperature of the injection system was set at 175 °C. The FID gas flows were: H_2: 40 ml min^{-1} and air: 400 ml min^{-1}. Optimization of these parameters was achieved using an authentic sample of **1**; this ensured that no thermal degradation of the neolignan occurred on the GC column. Low resolution MS were obtained at 70 eV and the spectra compared with an internal database. The MS of **1** and piperonyl alcohol **3** were obtained in similar GC–MS conditions, for the purpose of comparison.

3. Results and discussion

As we already observed [15,18,19], compound **1**, ingested at 100 μg ml^{-1} blood, showed neither feeding inhibition nor toxicity (less then 10% lethality) on fourth-instar larvae of *R. prolixus*. The drug treatment reduced the ecdysis by

30% and induced a slight delay of the moult. Also, it caused a significant reduction of the diuresis (83%) for a period of 6–24 h after feeding. In order to study the fate of burchellin in *R. prolixus*, hemolymph was analysed *in natura* by GC–MS. In the first experiment, the hemolymph was collected after 30 min of the end of the bloodmeal. The GC trace showed essentially four peaks (a_1–d_1) with retention times 3.6, 6.3 (major), 20.3 and 21.3 min respectively (Table 1). The latter peak (d_1) corresponded to intact burchellin identified by its RT and MS, both identical to those of an authentic sample of 1, recorded in the same experimental conditions. The first (a_1) and third (c_1) peaks (RT = 3.6 and 20.3 min) were present in very minute amounts. Peak a_1 was identified as aniline (**2**) on the basis of its MS matching by more than 90% with that deposited in the equipment library under the number CAS 000062-53-3. Peak c_1 could not be identified in this experiment due to the low quality of its MS. Nevertheless, its presence in the hemolymph after 30 min of the end of the bloodmeal, indicated that it is rapidly produced in the insect. This compound was identified in the second experiment (see below). Finally, peak b_1 showed no molecular ion in MS (highest *m/z* 135 $M^{\cdot+}$–OH). The presence of ions at *m/z* 91 (tropylium ion), 77, 65 and 51 was consistent with a benzyl compound. The ion at *m/z* 135 ($C_8H_7O_2$) confirmed this statement and allowed characterization of a piperonyl moiety. All this suggested the identification of b_1 as piperonyl alcohol (**3**). This hypothesis was further corroborated through co-injection of the hemolymph with an authentic sample of **3** prepared by $NaBH_4$ reduction of commercial piperonal **4** in 10% aq. THF, at r.t. Production of **3** in the insect results from the cleavage of the furan ring and loss of the semi-quinone part of burchellin **1**, as represented in Fig. 1 (steps a,b). Interestingly, a similar degradation sequence had been suggested by Gottlieb et al. [20] to explain the MS of burchellin itself [20].

In the second experiment, the hemolymph was collected after 1 h. Its GC trace was somewhat more complex. Table 1 reports the principal data obtained by GC–MS analysis. Eleven peaks (a_2–k_2) were observed. Among these, peaks a_2, b_2, i_2 and j_2 corresponded respectively to peaks a_1–d_1 discussed above. Piperonyl alcohol (peak b_2) is again the major compound. Aniline (peak a_2) is now present in much higher amounts than after half an hour of the bloodmeal (1st experiment) and although aniline seems to be unrelated to burchellin degradation, it accumulates with time in the hemolymph, either under **1** stress or as a common metabolite of the bloodmeal digestion. Similarly, peaks c_2 and h_2 are unrelated to degradation of **1**. The former (c_2) has been identified as 7-methyl-4-decene (**5**) by careful analysis of MS ($M^{\cdot+}$ 154 and fragment ions) and comparison of the MS data with a library of standards. The latter (h_2) is a MeOH adduct of **5**, $M^{\cdot+}$ 186 $C_{12}H_{26}O$ (Table 1).

Peak i_2 (referred to as peak c_1 in the first experiment) was now obtained in sufficient amount so that a clean MS could be recorded. The molecular ion (*m/z* 300) pointed to the general formula $C_{17}H_{16}O_5$, which corresponded to the loss of the allyl moiety of **1** as an allene group. Both the fragment ions at *m/z* 285 (loss of a methyl group), 267 (loss of water from the former ion), 239 (loss of CO from 267), and the ions indicating the piperonyl unit (*m/z* 135, 91, 77 and 51) confirmed

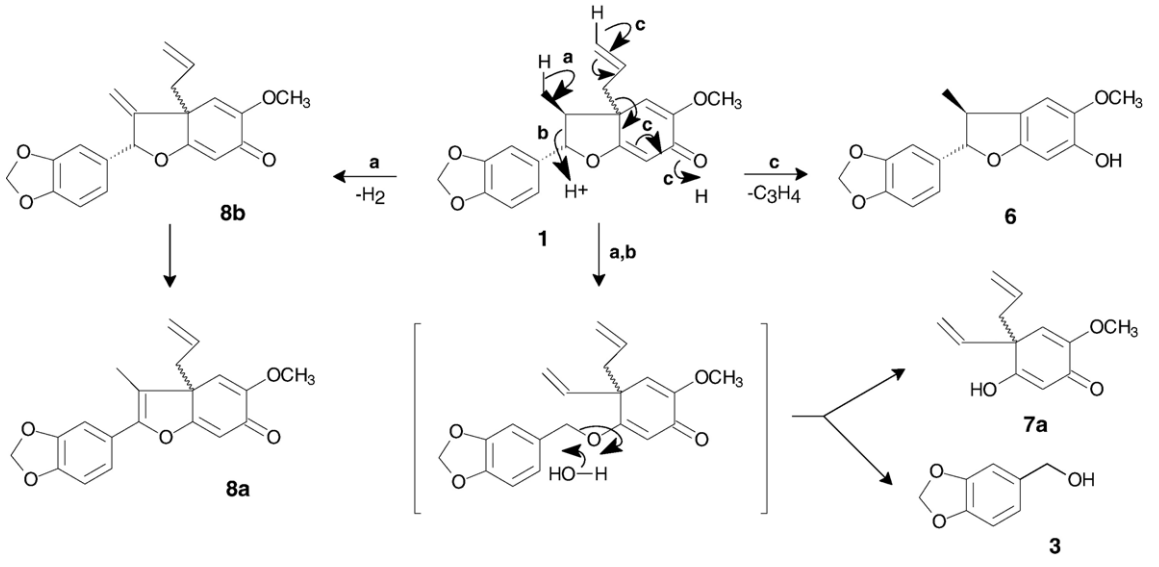

Fig. 1. Some steps of the degradation of burchellin (**1**) in the insect *R. prolixus*.

structural hypothesis **6**. The driving force for this biotransformation may be seen in the aromatization of the semiquinone of **1** into the methylcatechol moiety of **6** (Fig. 1, steps c). Again, this structure corresponds to an ion proposed earlier in the interpretation of the MS of **1** [20]. Since it had been established that **1** was stable under adopted GC conditions, it seems that *in vivo* degradation of **1** may follow some steps proposed for the electron impact induced degradation in the ionizing chamber of the mass spectrometer and, hence, that biological degradation in the insect parallels to some extent the physico-chemical degradation in the MS equipment.

The MS of peak d_2 showed a molecular ion at *m/z* 206, corresponding to general formula $C_{12}H_{14}O_3$, compatible with 6 degrees of unsaturation. The number of hydrogens present in this metabolite indicated that it contained the semiquinone unit and at least some of its substituents, *i.e.* the allyl and methoxyl groups, and part of the furan ring. This metabolite is thus the second fragment obtained when the heterocycle of burchellin **1** is cleaved, giving rise to **3** (Fig. 1, step a,b). Several structures (**7a**–**c**) can account for the observed general formula (Fig. 2). The extremely low amounts of catabolites of burchellin present in the hemolymph of the insect precluded the obtention of complementary spectral data; hence, it should be premature to choose one among shown alternatives.

The MS of peak k_2, $M^{\cdot+}$ 338 ($C_{20}H_{18}O_5$) indicated a dehydro derivative of burchellin. There are only two ways to introduce a double bond in **1**, both at the furan ring: endo (**8a**) or exocyclic to it (**8b**). The endocyclic alternative (**8a**) should be preferred for being more stable, as the supplementary double bond is tetrasubstituted and conjugated to the aromatic ring (Fig. 1, step a).

Three supplementary catabolites were present in extremely low amounts (peaks e_2–g_2). Their MS possess an ion at *m/z* 135, and may therefore contain the piperonyl moiety. Acceptable molecular formulas are: $C_{14}H_{18}O_3$, $C_{14}H_{16}O_3$ and $C_{14}H_{16}O_4$. All these metabolites seem to have undergone rearrangement at the furan ring, but in the absence of high resolution MS data, no reasonable structural proposal can presently be formulated.

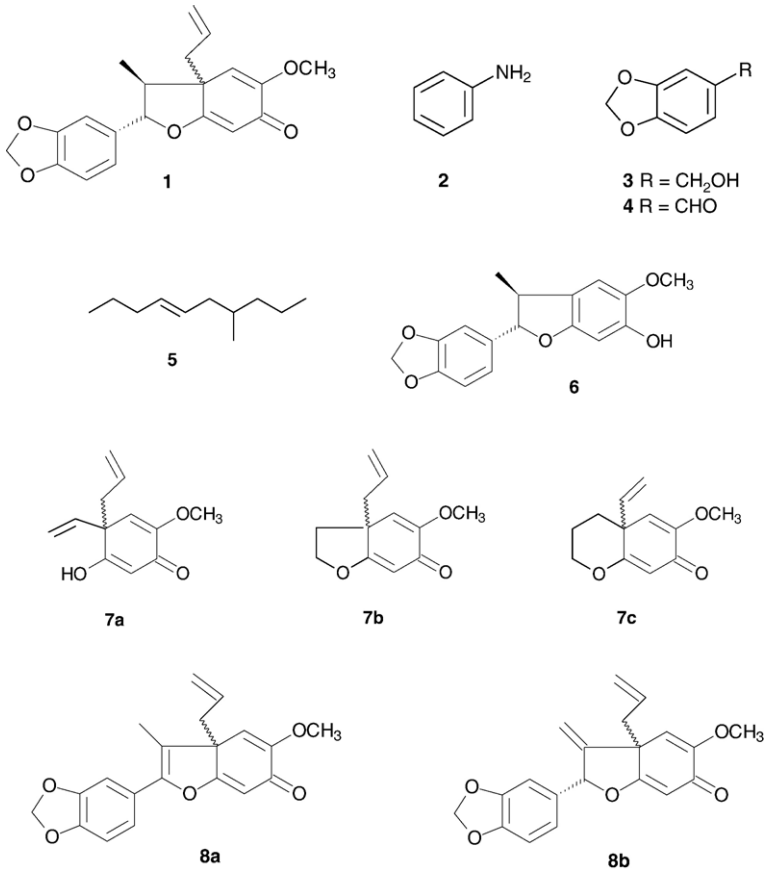

Fig. 2. Structure of compounds **1**–**8b**.

4. Conclusions

Burchellin is rapidly metabolized in the hemolymph of *R. prolixus*. Several ways of degradation can be proposed. The elimination of the allyl group may occur as the first step, but degradation takes place, principally, at the furan ring and produces one major metabolite, identified as piperonyl alcohol. **3** is formed in the early steps of burchellin degradation, accumulates in the hemolymph and may be directly involved in the inhibition of urine secretion. This hypothesis is now being investigated looking for bioactivity of piperonyl alcohol itself and of a series of derivatives.

Interestingly, piperonal (**4**) (Fig. 2) has been reported to be a home repellent of louse [22]. More recently, Harmatha and Nawrot described the insect feeding deterrent activity of lignans containing a piperonyl structure moiety, and of some methylenedioxy-containing simple phenolics, including piperonal [23]. The authors concluded that, although the simple phenolics showed low activities as feeding deterrents, the presence of a piperonyl moiety clearly resulted in increased antifeedant activity of the lignans. Accordingly, our results also implicate the piperonyl moiety in biological activity on insects. Further work is needed to understand the involvement of piperonyl-containing structures on physiological processes in insects.

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