Tonantzitlolones from Stillingia lineata ssp. lineata as potential inhibitors of chikungunya virus

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ABSTRACT

With the purpose of discovering new chemical classes of molecules, in particular those with selective antiviral activity, extracts from plants growing in Reunion Island were systematically evaluated in a chikungunya virus-cell-based assay. The entire ethyl acetate extract obtained from the stem bark of *Stillingia lineata* ssp. *lineata* (Euphorbiaceae) exhibited selective antiviral activity against the chikungunya virus with an $EC_{50} < 0.8 \ \mu g/mL$, whereas only a weak cytotoxic effect was observed on the host cells. A phytochemical investigation of this extract led to the isolation of tonantzitlolone A and tonantzitlolone B (**1** and **2**), together with the new 4′-hydroxytonantzitlolone, named tonantzitlolone C (**3**), which has an uncommon C15-flexibilane skeleton, as well as in the new *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**). Subsequent evaluation for inhibition of chikungunya virus replication *in cellulo* demonstrated that the 4′-acetoxytonantzitlolone (**2**) was endowed with antiviral activity against CHIKV.

1. Introduction

Chikungunya is an arboviral disease that is typically characterized by myalgia, polyarthralgia, fever, nausea, headaches and maculopapular rash. The chikungunya virus (CHIKV) is an *Aedes* mosquitoes-transmitted alphavirus that belongs to the Togaviridae family (Kaur and Chu, 2013). Around a decade ago, there was a sudden reemergence of CHIKV infections with a previously unseen magnitude, with the virus moving from Africa throughout the Indian Ocean to India and Southeast Asia. Due to the steadily increasing number of cases, it became a serious concern and threat for public health (Thiberville et al., 2013). In 2005 and 2006, CHIKV caused a massive outbreak on Reunion Island, a French overseas territory, during which about 266,000 of the 775,000 inhabitants became infected (Renault et al., 2007). This was the dawn of a worldwide emergence of this virus, which has now even reached the Caribbean, the doorstep to the rest of the Americas. At present, no vaccine is yet available to prevent this infection, nor any antiviral drug(s) for treatment or prophylaxis.

The Reunion Island outbreak triggered our interest to explore the vast Indian Ocean plant biodiversity for novel natural molecules that selectively inhibit the replication of CHIKV. More than 1500 extracts from 972 endemic or indigenous plants from Madagascar, Mauritius and Reunion Island were evaluated for selective antiviral activity in a virus-cell-based assay for CHIKV (Leyssen et al., 2014).

Among them, the species *Croton mauritianus* (Euphorbiaceae) from Reunion Island was found to elicit biological activity and was selected for a phytochemical investigation, leading to the isolation of tigliane-type diterpenoids (Corlay et al., 2014). Tigliane-, daphnane- and jatrophane-type diterpenoids with potent and selective anti-CHIKV activities were also isolated from other Euphorbiaceae species: *Trigonostemon cherrieri* (Allard et al., 2012a,b; Bourjot et al., 2014), which is native to New Caledonia, *T. howii* (Bourjot et al., 2012), which is native to Vietnam, and the Corsican species *Euphorbia amygdaloides* ssp *semiperfoliata* (Nothias-Scaglia et al., 2014). These results prompted us to investigate another Euphorbiaceae species growing in Reunion Island, *in casu Stillingia lineata* ssp. *lineata,* of which the stem bark extract showed potent and selective anti-CHIK activity.

The genus *Stillingia* comprises 30 species that are mainly distributed in neotropical areas. In 1980, eight daphnane- and

tigliane-type diterpenoids were isolated from roots of *Stillingia sylvatica* (Adolf and Hecker, 1980). Cembrene A, two novel diterpenes with rare flexibilane skeletons (tonantzitlolone A and tonantzitlolone B), two new pimaranes, seven kauranes, three known atisanes, a new trachylobane, and the new pentacyclic diterpene sanguinolane with a novel skeleton have been reported from the roots of *Stillingia sanguinolenta* (Dräger et al., 2007).

Cytotoxic, proteolytic and antifungal activities have been reported for several species (Adolf and Hecker, 1980; Chapuis et al., 1988; Cota et al., 2011; Sequeiros et al., 2003). The *in vitro* cytotoxic activity of the species *Stillingia lineata* (Lam.) Müll. Arg. from Mauritius was evaluated on human tumor cells Co-115 (colon cancer), with petroleum ether and DCM leaf extracts that were highly cytotoxic (Chapuis et al., 1988). This species has two subspecies: the ssp. *pacifica* which is located in Malaysia, and the ssp. *lineata,* which is endemic to Mauritius and to Reunion Island. The latter is a medicinal plant that has been traditionally used in Mauritius for diabetes (Picot et al., 2014).

This study reports the bio-assay-guided fractionation of the EtOAc bark extract from *S. lineata* ssp. *lineata* in a virus-cell-based assay for CHIKV on Vero cells (green monkey kidney cells *Cercopithecus aethiops*), as well as the characterization of the purified diterpenes–the most predominant secondary metabolite in this plant species–by detailed analysis of their spectral data, using 500 MHz 2D-NMR (COSY, NOESY, HSQC, HMBC) and mass spectrometry.

2. Results and discussion

A total of 320 EtOAc polyamide cartridge-filtered extracts were prepared from different parts of 171 Reunion Island plant species and were evaluated for selective antiviral activity in a CHIKV virus-cell-based assay (Leyssen et al., 2014). The results of this screening indicated that the EtOAc extract that was obtained from eight species completely inhibited the virus from inducing cytopathic effects and without showing adverse effects on the host cell morphology and density compared to the untreated, uninfected cell control condition (EC_{50} from <0.8 to 9.1 μg/mL).

Among them, the *S. lineata* ssp. *lineata* EtOAc bark extract (Euphorbiaceae) was selected for its potent anti-CHIKV activity (EC_{50} < 0,8 μg/mL) and weak cytotoxicity on Vero cells (CC_{50} = 60.9 μg/mL). This EtOAc extract was subjected to silica gel flash chromatography to give six fractions. The biologically active fractions F3, F4 and F5 (EC_{50} = 7.23, 2.01 and 2.05 μg/mL, respectively) were further purified by preparative and semi-preparative C18HPLC to yield three rare macrocycle C15-type diterpenes: tonantzitlolone A, tonantzilolone B and 4′-hydrox-ytonantzitolone (compounds **1–3**), and also *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**), a new pimarane Fig. 1.

The total assignment of ^{1}H and ^{13}C NMR data of tonantzi-tlolones **1, 2** and **3** and compound **4** are presented in Tables 1 and 2.

Compound **1** was isolated as a white powder. The HR-ESIMS indicated a $[M + Na]^+$ ion peak at 487.2688, which suggested a molecular formula of $C_{26}H_{40}O_7Na$ (calc. 487.2672), thus requiring seven double-bond equivalents. Compound **2** was obtained as an amorphous powder. The HR-ESIMS of the $[M + Na]^+$ ion peak at *m/z* 545.2717 suggested that the molecular formula of compound **2** as $C_{28}H_{42}O_9$ (calculated for $C_{28}H_{42}O_9Na$ 545.2727), thus requiring eight double-bond equivalents. Considering the biological source, the ^{1}H and ^{13}C NMR spectroscopic data suggested that compounds **1** and **2** possessed the rare macrocycle C15-flexibilane skeleton and were identified through comparison with literature data as tonantzitlolone A and tonantzitlolone B (4′-acetoxytonantzitlo-lone) (Dräger et al., 2007; Wittenberg et al., 2004). Both compounds possess characteristic NMR features of these flexibi-lane-type diterpenoids, in particular a tertiary and a secondary hydroxy group (OH-9 and OH-10) and two tertiary methyl groups (C-19 and C-20). Amongst the four quaternary carbons of the macrocycle were found a keto group (C-4), a non-functionalized carbon (C-15)–already mentioned for the flexibilene (Kazlauskas et al., 1978)–two deshielded carbons (C-9 and C-11), an acetal, and an oxygen-bearing carbon. The same deshielding was observed for the tertiary carbons of these oxygenated bridges (C-5 and C-14) as was found with other CH carbons. More particularly, the presence of the very deshielded C-9 flanked by two oxygen atoms was determined by chemical shift (δC 97) and by the ^{2}J HMBC correlation from OH-9. The location of this acetal C-9 was confirmed by multiple HMBC correlations (for example, chemical shifts of compound **1**) from H-8 (δH 4.89), H-10 (δH 3.43), OH-10 (δH 3.10) and H-5 (δH 4.62), fixing the ether-ring position.

The X-ray diffraction analysis from crystals of compound **1** allowed us to confirm the absolute configuration of tonantzitlolone A that was previously established by Dräger and his colleagues as 3S,5R,7R,8R,9R,10S,11R,14S (Dräger et al., 2007). A perspective
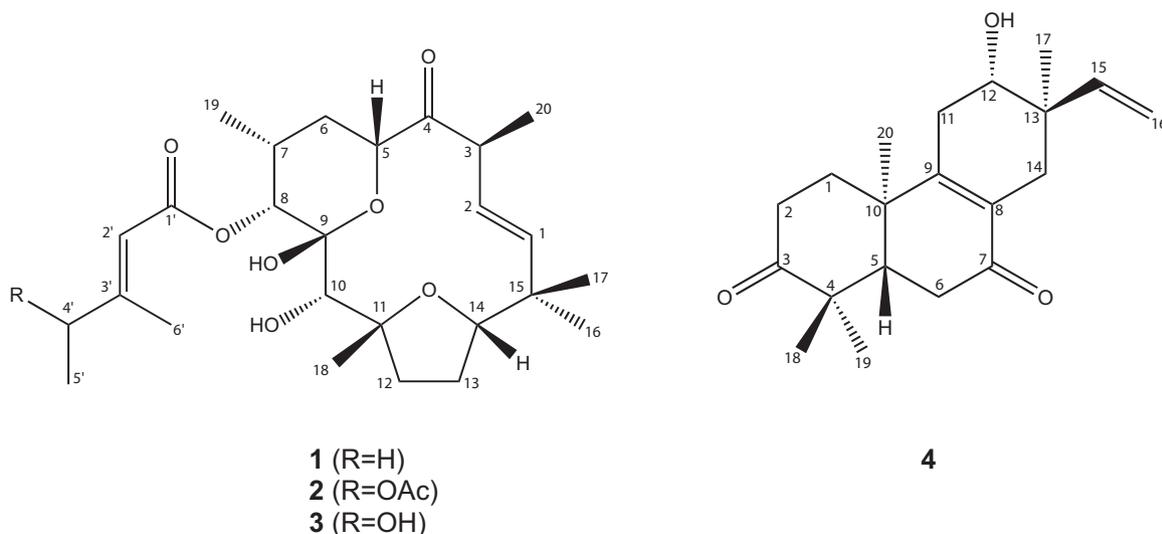

1 (R=H)
2 (R=OAc)
3 (R=OH)

4

Fig. 1. Compounds isolated from *S. lineata* ssp. *lineata* stem bark: tonantzitlolone (**1**), 4′-acetoxytonantzitlolone (**2**), 4′-hydroxytonantzitlolone (**3**) and *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**).

ORTEP plot is shown in Fig. 2. CCDC 1033736 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retriev-ing.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; or deposit@ccdc.cam.uk).

The three-dimensional structure of 4′-acetoxytonantzitlolone (tonantzitlolone B) (**2**) does not differ from that of tonantzitlolone A. Its absolute configuration is, however, known through the work of Busch and his colleagues, in which the *R* configuration was demonstrated (Busch et al., 2008).

Compound **3** possessed the molecular formula $C_{26}H_{40}O_8$, based on its $[M+Cl]^-$ ion peak at m/z 515.2410, obtained by HR-ESIMS (calculated for $C_{26}H_{40}O_8Cl$ 515.2412), thus implying seven double-bond equivalents. In agreement with the mass spectrum, the ^{13}C NMR spectrum indicated the presence of 26 carbon atoms. The chemical shifts and the DEPT135 spectrum allowed us to highlight the presence of six quaternary carbons (one ketone carbonyl, one carbonyl ester, one olefinic, one oxygenated and two non-functionalized), three methylenes, seven methyls, and ten methines (three olefinic and five oxygenated). The ^1H and ^{13}C NMR spectra (Table 1) of compound **3** were very similar to those of the known compounds **1** and **2**, thus one could deduce that the structure of compound **3** was a tonantzitlolone derivative. All the ^{13}C NMR signals of compound **3** were reminiscent to those of compound **2**, except for carbons C-2′–C-5′ (δC 113.3, 162.8, 72.4 and 21.5, respectively for compound **3**, and 115.2, 157.6, 73.8 and 19.1, respectively for compound **2**), suggesting the modification of the side chain. In the ^1H NMR and HSQC spectra, the presence of a deshielded quartet of doublets at δH 4.62 (*J* = 6.5, 2.5 Hz)

attributable to proton H-4′, attached to C-4′ (δC 72.4), suggested the presence of the —CH(OH)-CH₃ moiety. The HMBC correlation from this proton at δH 4.26 to the olefinic and methyl carbons at δC 113.3 (C-2′) and 15.3 (C-6′) confirmed that the —CH(OH)-CH₃ moiety was located at C-3′. Consequently, the planar structure of compound **3** was elucidated as 4′-hydroxytonantzitlolone, named tonantzitlolone C. The relative configuration of compound **3**, with the exception of the stereocenter C-4′, could be determined through the analysis of NOESY correlations, which are all comparable to those of compounds **1** and **2**. In addition, since the specific rotation value of compound **3** ($[\alpha]^{25}_D + 76$ [*c* 0.25, CHCl₃]) was found to have the same sign as the value found for tonantzitlonone A ($[\alpha]^{25}_D + 134$ [*c* 0.25, CHCl₃]), it could be postulated that they possess the same absolute configuration (Dräger et al., 2007).

Compound **4** was obtained as an amorphous powder. The HR-ESIMS of the $[M+H]^+$ ion peak at *m/z* 317.2131 allowed us to establish its molecular formula as $C_{20}H_{28}O_3$ (calculated for $C_{20}H_{29}O_3$ 317.2117), thus requiring seven double-bond equivalents. Its IR spectrum showed characteristic absorption bands at 3457 cm⁻¹ for the hydroxy group, and 1707 and 1663 cm⁻¹ for the carbonyl groups. The maximum absorption bands in the UV spectrum at 243 nm indicated the presence of an α,β-unsaturated carbonyl. The occurrence in the ^1H NMR spectrum (Table 2) of a vinyl group with three dd resonances at δH 5.74 (*J* = 17.5 and 10.8 Hz, H-15), δH 5.22 (*J* = 10.8 and 1.0 Hz, H-16*cis*), and δH 5.18 (*J* = 17.5 and 1.0 Hz, H-16*trans*) and the presence of four methyl group singlets suggested a pimarane-type skeleton. The analyses of ^1H, ^{13}C, DEPT135, HMBC and HSQC NMR data revealed that compound **4** contains 20 carbons, including four singlet methyls,

Table 1
^1H and ^{13}C NMR spectroscopic data for tonantzitlolones (**1–3**) (in CDCl₃, at 300 and 75 MHz, respectively ; δ in ppm).
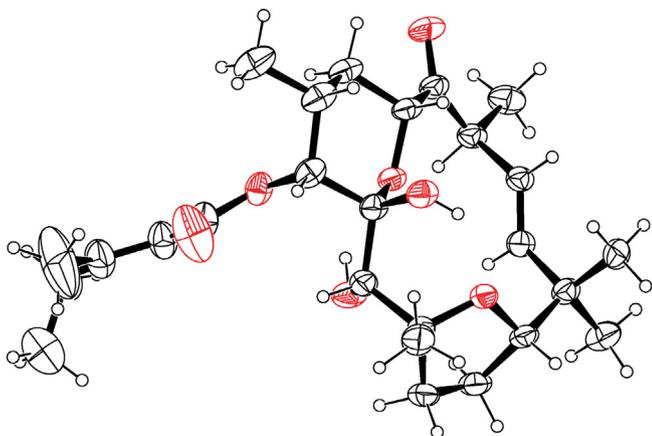
Position	1		2		3	
	δ_C	δ_H, mult, J (Hz)	δ_C	δ_H, mult, J (Hz)	δ_C	δ_H, mult, J (Hz)
1	140,1	5,85 (d, 1H, 15.5)	140,1	5,85 (d, 1H, 15.0)	140,0	5,85 (d, 1H, 15.5)
2	126,8	5,23 (dd, 1H, 15.5, 9.5)	126,8	5,24 (dd, 1H, 15.5, 9.5)	126,8	5,23 (dd, 1H, 15.5, 9.5)
3	49,5	3,33 (dq, 1H, 9.5, 7.0)	49,5	3,32 (dq, 1H, 9.5, 7.0)	49,5	3,34 (dq, 1H, 9.5, 7.0)
4	211,3	–	211,2	–	211,2	–
5	74,1	4,62 (dd, 1H, 12.0, 3.0)	74,1	4,62 (dd, 1H, 12.0, 3.0)	74,1	4,62 (dd, 1H, 12.0, 3.0)
6α	28,8	1,84 (dt, 1H, 13.5, 3.5)	28,7	1,84 (dt, 1H, 13.5, 3.5)	28,8	1,85 (ddd, 1H, 13.0, 3.0, 2.5)
6β		1,40 (ddd, 1H, 13.5, 12.5, 3.0)		1,40 (ddd, 1H, 13.5, 12.5, 3.0)		1,39 (ddd, 1H, 13.5, 12.5, 3.0)
7	29,0	2,33 (dddq, 1H, 12.5, 7.0, 4.0, 2.5)	29,0	2,32 (dddq, 1H, 12.5, 7.0, 4.0, 2.5)	29,0	2,33 (dddq, 1H, 11.5, 7.0, 4.0, 2.5)
8	73,2	4,89 (d, 1H, 2.5)	73,7	4,90 (d, 1H, 2.5)	73,5	4,90 (d, 1H, 2.5)
9	97,1	–	97,1	–	97,0	–
10	78,2	3,43 (d, 1H, 6.0)	78,1	3,40 (br.d, 1H, 6.0)	78,2	3,41 (br.d, 1H, 2.5)
11	87,6	–	87,7		87,6	–
12α	37,3	1,53 (ddd, 1H, 12.5, 12.5, 7.5)	37,3	1,53 (ddd, 3H, 12.5, 12.5, 7.5)	37,3	1,53 (ddd, 1H, 12.5, 12.5, 7.5)
12β		2,44 (dd, 1H, 12.5, 7.5)		2,43 (dd, 1H, 12.5, 7.5)		2,41 (dd, 1H, 12.5, 7.5)
13α	28,0	1,76 (ddd, 1H, 12.5, 7.5, 5.0)	28,0	1,76 (ddd, 1H, 12.5, 7.5, 5.0)	28,0	1,76 (ddd, 1H, 12.5, 7.5, 5.0)
13β		2,04 (dddd, 1H, 12.5, 12.5, 12.5, 7.5)		2,03 (dddd, 1H, 13.0, 13.0, 11.5, 7.5)		2,03 (dddd, 1H, 11.5, 11.5, 11.5, 7.5)
14	88,9	3,76 (dd, 1H, 11.5, 5.0)	88,9	3,76 (dd, 1H, 11.5, 5.0)	88,9	3,76 (dd, 1H, 11.5, 5.0)
15	38,7	–	38,7	–	38,7	–
16	25,4	0,91 (s, 3H)	25,4	0,91 (s, 3H)	25,4	0,91 (s, 3H)
17	25,1	1,14 (s, 3H)	25,1	1,13 (s, 3H)	25,1	1,14 (s, 3H)
18	28,1	1,37 (s, 3H)	28,1	1,37 (s, 3H)	28,1	1,37 (s, 3H)
19	17,0	0,84 (d, 3H, 7.0)	17,0	0,84 (d, 3H, 7.0)	17,0	0,85 (d, 3H, 7.0)
20	16,0	1,11 (d, 3H, 7.0)	16,0	1,12 (d, 3H, 7.0)	16,0	1,12 (d, 3H, 7.0)
1′	166,6	–	166,3	–	166,5	–
2′	113,9	5,69 (q, 1H, 1.5)	115,2	5,90 (q, 1H, 1.2)	113,3	6,00 (s, 1H)
3′	163,0	–	157,6		162,8	–
4′	33,9	2,17 (q, 2H, 7.0)	73,8	5,27 (q, 1H, 6.5)	72,4	4,62 (dq, 1H, 6.5, 2.5)
5′	11,8	1,07 (t, 3H, 7.5)	19,1	1,35 (d, 3H, 7.0)	21,5	1,32 (d, 1H, 6.5)
6′	19,0	2,15 (d, 3H, 1.5)	15,2	2,13 (d, 3H, 1.5)	15,3	2,12 (s, 1H)
1″			170,1	–		
2″			21,2	2,09 (s, 3H)		
OH-4′						Not resolved
OH-9		5,65 (s, 1H)		5,70 (s, 1H)		5,63 (s, 1H)
OH-10		3,10 (d, 1H, 6.0)		3,05 (br.d, 1H, 6.5)		3,15 (d, 1H, 5.5)

Table 2
^{1}H and ^{13}C NMR spectroscopic data for *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**) (in CDCl$_3$, at 500 and 75 MHz, respectively).

Position	δ_C	δ_H, mult., (J en Hz)	NOESY[a]
1α	34,5	2,17 (ddd, 1H, 13.0, 6.0, 3.0)	2α,20
1β		1,78 (td, 1H, 13.0, 6.0)	2β, 5
2α	34,6	2,74 (ddd, 1H, 16.0,13.0, 6.0)	1α, 19, 20
2β		2,48 (ddd, 1H, 16.0, 6.0, 3.0)	1β
3	218,8	–	–
4	46,5	–	–
5	49,8	2,20 (dd, 1H, 14.0, 4.0)	1β11β, 18
6α	35,4	2,53 (dd, 1H, 17.5, 14.0)	19, 20
6β		2,46 (dd, 1H, 17.5, 4.0)	18
7	204,8	–	–
8	133,0	–	–
9	160,8	–	–
10	38,8	–	–
11α	30,3	2,22 (dd, 1H, 17.5, 9.0)	17
11β		2,58 (dd, 1H, 17.5, 5.5)	5, 12β, 14β
12β	71,0	3,61 (dd, 1H, 9.0, 5.5)	11β, 14β, 15
13	40,0	–	–
14α	34,2	2,35 (d, 1H, 17.0)	17
14β		2,23 (d, 1H, 17.0)	11β, 12β, 15
15	144,7	5,74 (dd, 1H, 17.5, 10.8)	12β, 14β
16cis	115,5	5,22 (dd, 1H, 10.8, 1.0)	–
16trans		5,18 (dd, 1H, 17.5, 1.0)	17
17	15,0	0,93 (s, 3H)	11α, 14α, 16trans
18	25,2	1,11 (s, 3H)	5, 6β
19	21,4	1,14 (s, 3H)	2α, 6α, 20
20	17,3	1,30 (s, 3H)	1α, 2α, 6α, 19
OH		2,24 (br.s, 1H)	–

[a] Key NOESY cross peaks.

six methylenes (one sp^2 and five sp^3), three methine (one sp^2 and two sp^3), seven quaternary carbons (one α,β-unsaturated carbonyl, and one oxygenated sp^2, two sp^2 and three sp^3). The key 2D NMR correlations of compound **4** are presented in Fig. 3. The relative configuration of compound **4** was deduced from ^{1}H–^{1}H vicinal coupling constants and NOESY correlations. Cross peaks observed between Me-20 and Me-19, H-1α, H-2α and H-6α suggested that they are cofacial and, thus indicated that Me-20 is α-oriented as observed for an *ent* structure. The relative configuration of C-5 was established by the correlations between H-5 and Me-18 on the one hand, and between H-5 and H-1β, on the other hand, which is consistent with a β-orientation. This stereochemistry was also supported by the axial–axial coupling constants shown by both H-5 and H-6α (Table 2). The hydroxymethine proton H-12 resonated as a dd at δH 3.61 with coupling constants of 9.0 and 5.5 Hz. The magnitude of these coupling constants suggests the axial position for this proton. Moreover both H-12 and H14β are cross-correlated and both are also correlated with H-15 and H-11β.

Fig. 2. An ORTEP-3 view of the tonantzitlolone A (**1**). Ellipsoids are drawn at the 30% probability level.

Thus, the anti-position of H-15 compared to Me-20 suggests an isopimarane structure. In addition, the negative value of the optical rotation (−225, CHCl$_3$, c = 0.20 mg/mL) of compound **4** is the same sign as those measured for other *ent*-pimarane derivatives (Luo et al., 2001; Rayanil et al., 2013). Compound **4** was, therefore, elucidated as *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene.

It should be emphasized that the macrocycle-15C diterpene skeleton of compounds **1**, **2** and **3** is particularly unusual in natural products. To the best of our knowledge, this constitutes the third example of tonantzitlolone-type diterpenes isolated from higher plants. The tonantzitlolone A (**1**) was isolated for the first time by Dräger et al. (2007) from *Stillingia sanguinolenta*, an endemic species of Mexico, and later on from *Sebastiania macrocarpa* (Euphorbiaceae) (Lima et al., 2009). These compounds–including tonantzitlolone B (**2**), which was also isolated from *Stillingia sanguinolenta* (Dräger et al., 2007)–have not yet been identified in other plants. The tonantzitlolone C (4'-hydroxytonantzitlolone (**3**)) represents the third natural compound of this chemical series that possesses a 15C-macrocycle flexibilen carbon skeleton. This original skeleton is also found in the structure of flexibilen, which was isolated from the soft coral *Sinularia flexibilis* (Hérin et al., 1976; McMurry et al., 1982). Unlike tonantzitlolones, the pimaranes have been isolated from different genera and families. Two pimaranes were previously isolated from *S. sanguinolenta*: the *ent*-8β-hydroxy-3,12-dioxopimara-9(11), 15-diene and the *ent*-3β,12α-8-dihydroxypimara,15-diene, which belong to the enantiomeric series (Dräger et al., 2007).

The antiviral properties of the isolated compounds were evaluated in a CHIKV virus-cell-based assay, with chloroquine as a reference compound. Only the 4'-acetoxytonantzitlolone (**2**) selectively inhibited virus-induced cell death, with EC$_{50}$ and SI (selectivity index, calculated as CC$_{50}$ Vero/EC$_{50}$ CHIKV) values of 7 μM and 8.8, respectively, while 4'-hydroxytonantzitlolone (**3**) was not significantly selective (EC$_{50}$:34 μM; SI:3.2). A comparison of the structures of these two bioactive compounds reveals that the presence of an acetyl group is possibly an important element in improving the inhibitory activity.
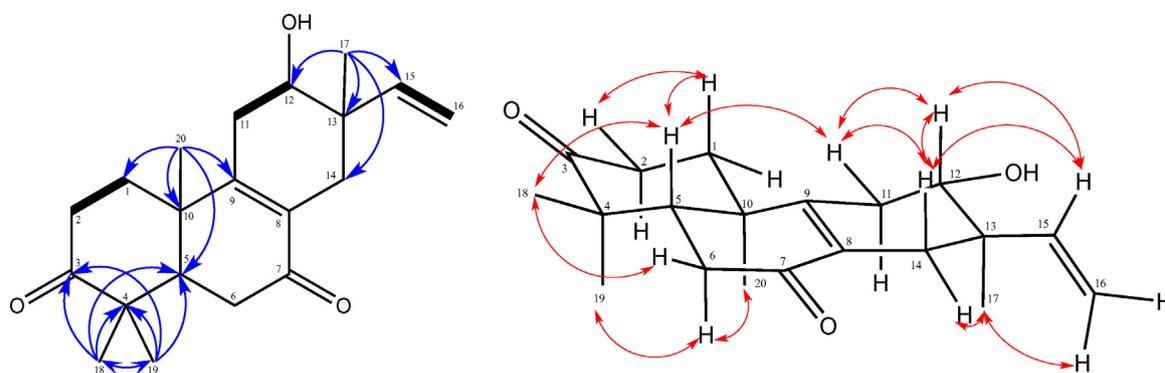
Fig. 3. Selected COSY (bold), HMBC (blue arrows) and NOESY (red arrows) correlations for structural elucidation of *ent*-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In conclusion, the bioguided fractionation of the bark of *Stillingia lineata* ssp. *lineata* resulted in the isolation and characterisation of two new diterpenes (**3** and **4**), together with two known molecules (**1** and **2**). Their structures were elucidated mainly by 2D-NMR techniques and mass spectrometry. The isolated molecules were only slightly active against CHIKV in their purified state. The more pronounced antiviral activity of the extract could be due to additive or synergistic effects by other compounds that are present in low amounts. The correlation between the structure and biological activity of these tonantzi-tlolones is not yet complete. Therefore, further investigation of these active diterpenes in the context of CHIKV is required not only to elucidate the structure–activity relationship but also to enable the study of the molecular mechanism of action.

3. Experimental

3.1. General

Optical rotations were measured using a Jasco P1010 or an Anton Paar MCP200 polarimeter. UV spectra were acquired on a Varian Cary 100 Scan spectrophotometer. IR spectra were recorded on a PerkinElmer Spectrum FT-IR 100 spectrophotometer. NMR spectra were recorded on a Bruker Avance 300 MHz or 500 MHz instrument at 25 °C. ^{1}H- and ^{13}C NMR chemical shifts refer to CHCl$_3$ at 7.26 ppm, and to CDCl$_3$ at 77.0 ppm, respectively. Coupling constants are in Hz. HR-ESIMS data were obtained on a Thermoquest TLM LCQ Deca ion-trap spectrometer. X-ray crystallographic data were collected using a Rigaku diffractometer constituted by a MM007HF rotating-anode generator, equipped with Osmic confocal optics, delivering filtered Cu-Kα radiation (λ = 1.54187 Å) and a Rapid II curved Image Plate, at a cryogenic temperature (193 K).

Flash chromatographies were performed on a Sepacore system (Büchi) or a CombiFlash Companion apparatus (Teledyne Isco), using silica gel 60A or various prepacked normal-phase cartridges (Silicycle® 15–40 μm, Redisep® 15–60 μm, Redisep® 35–70 μm, or Versapack® 45–75 μm). All solvents were purchased from Carlo Erba (France). TLC separations were performed on Merck precoated silica gel 60G F254, Alugram® SIL G/UV254 (Macherey-Nagel) or Alugram® RP-18W/UV254 (Macherey-Nagel) analytical plates. Kromasil reversed-phase semi-preparative and preparative HPLC separations were carried out on Thermo Hypersil columns (250 × 10 mm and 250 × 21 mm ID, 5 μm), using Waters® apparatus (Alliance 2695 and Autopurification system Waters®–2767 Sample Manager) equipped with a binary pump (Waters 2525), a UV–vis diode array detector (190–600 nm, Waters® 2996), and a PL-ELS 1000 ELSD detector (Polymer Laboratory).

3.2. Plant material

The whole plant of *Stillingia lineata* ssp. *lineata* was collected in March 2011 in Langevin (La Réunion, France) and was authenticated by the botanist Prof. Dominique Strasberg (University of La Réunion). A voucher specimen (No. REU09532) was deposited in the Herbarium of the University of La Réunion, France.

3.3. Extraction and isolation

Seven hundred sixty-nine grams of dried powder of bark of *Stillingia lineata* ssp. *lineata* were extracted with EtOAc, using a Dionex-accelerated solvent extractor equipped with 100 mL of stainless steel cells and 250 mL of collection bottles (ASE300, Voisins Le Bretonneux, France). Conditions were as follows: temperature, 40 °C; pressure, 100 bars; 5 cycles with static extraction time of 6 min; and flush volume, 100%. The EtOAc crude extract was concentrated under reduced pressure to yield 33.9 g of residue (ρ = 4.4% w/w). This EtOAc extract was subjected to flash silica chromatography using a gradient of cyclohexane–AcOEt (95:5–0:100) and AcOEt–MeOH (100:0–50 :50) at 15 mL/min to produce six fractions, according to their TLC profiles. The biologically active fractions F3 (8.3 g, EC$_{50}$ = 7.23 μg/mL), F4 (4.7 g, EC$_{50}$ = 2.91 μg/mL), and F5 (3.5 g, EC$_{50}$ = 2.05 μg/mL) were further purified using successive preparative and semi-preparative chromatographies on reversed-phase silica gel in the C-18 column. Purifications of fraction F3 (8.3 g, cyclohexane–AcOEt 80:20) were carried out by preparative RP-18HPLC chromatography (Thermo-hypersil Kromasil, 5 μm, 250 × 21.2 mm) to produce tonantzitlolone A (**1**), using MeOH–H$_2$O + 0.1% formic acid (from 80:20 to 100:0 in 20 min at 21 mL/min ; t_R 18.9 min). Fraction F4 (4.7 g, cyclohexane–AcOEt 80:20) was separated by preparative HPLC (Kromasil RP-18 using MeOH–H$_2$O + 0.1% formic acid, from 70:30 to 100:0 in 30 min at 15 mL/min) to give the 4′-acetoxytonantzitlolone (**2**) (19.7 mg, t_R 20.3 min). The fraction F5 (3.5 g, eluting from 70:30 to 50:50 cyclohexane–AcOEt) was subjected to preparative RP-18HPLC to yield compound **3** (9.3 mg, t_R 17.2 min) and a sub-fraction 5–5 (20 mg). This sub-fraction 5–5 was further purified by a semi-preparative HPLC (Thermo Hypersil Kromasil, MeOH–H$_2$O + 0.1% formic acid, from 60:40 to 100:0 in 50 min at 4.7 mL/min) to get compound **4** (1.2 mg).

4′-Hydroxytonantzitlolone (tonantzitlolone C) (**3**)
White, amorphous powder; $[\alpha]^{23}_D$ + 76° (*c* 0.25, CHCl$_3$); UV (CHCl$_3$) λ$_{max}$ (log ε) 240 (2.46) nm; IR ν$_{max}$ (cm^{-1}): 3372, 2967, 2933, 1712; ^{1}H and ^{13}C NMR (CHCl$_3$) data, see Table 1; HR-ESIMS (neg.) *m/z* 515.2410 (calculated for C$_{26}$H$_{40}$O$_8$Cl, 515.2412).

Ent-12α-hydroxy-3,7-dioxoisopimara-8,15-diene (**4**)
White, amorphous powder; $[\alpha]^{23}_D$ − 225° (*c* 0.20, CHCl$_3$); UV (CHCl$_3$) λ$_{max}$ (log ε) 201 (1.83) 227 (2.01) 243 (2.36) nm; IR ν$_{max}$

(cm^{-1}): 3457, 2924, 1707, 1663, 1380; ^{1}H and ^{13}C NMR (CHCl$_3$) data, see Table 2; HR-ESIMS (pos.) m/z 317.2131 (calcd. for C$_{20}$H$_{29}$O$_3$, 317.2117).

3.4. X-ray crystallographic analysis of 1

A large colourless crystal of tonantzitlolone A (**1**), recrystallized from isopropyl oxide, was mounted at cryogenic temperature (193 K) on a Rigaku diffractometer constituted by a MM007HF rotating-anode generator, equipped with Osmic confocal optics, delivering filtered Cu-Kα radiation (λ = 1.54187 Å) and a Rapid II curved Image Plate. The crystal-to-detector distance was 127.40 mm. The X-ray data were collected up to a maximum 2θ value of 136.5°. Four images with one degree ω oscillation spanning a 60° ω angular space were used to derive cell constants and matrix orientation using d^*trek (Pflugrath, 1999). According to a subsequent strategy in mmm symmetry, a total of 108 oscillation images were collected from three sweeps of data using ω oscillations in 5.0° steps. The exposure rate was 60.0 s per degree. Data reduction and scaling were carried out using $Fs\_Process$.[2]. The structure was solved by Direct methods (SHELXS-97) and by subsequent difference Fourier syntheses, and refined by full matrix least squares on F^2 using the SHELXL-2014/6 (Sheldrick, 2008). Anisotropic thermal parameters were used for all non-hydrogen atoms whereas hydrogen atoms, located from difference Fourier maps, were refined as a riding model with U_{iso} = 1.2U_{eq} of the parent atom (1.5 for the methyl hydrogen atoms and the hydroxyl hydrogen ones). The C28 methyl carbon atom went to non-positive-definite and was further restrained with a soft ISOR (esd 0.04) restraint to be approximately isotropic. Despite the absence of strong anomalous scatterers (Friedif value of 34 (Flack and Shmueli, 2007)), the absolute configuration was attributed with some confidence by the use of the Hooft y parameter (Hooft et al., 2008), calculated as 0.03 (5), the P2(true) and P3(true) values being both 1.000, from a total of 1979 Bijvoet pairs (97% coverage). This Bayesian analysis on the Bijvoet pairs implemented in Platon (Spek, 2009) enhanced the weak significance of the Flack x parameter (Flack, 1983), which was determined to be x = 0.1 (3) by the use of 1519 quotients $[(I+) - (I-)]/[(I+) + (I-)]$ from the Parsons' method (Parsons et al., 2013)

The crystallographic data are summarized as follows: a colourless tab with dimensions 0.60 × 0.48 × 0.12 mm, C$_{26}$ H$_{40}$ O$_7$, Mr = 464.58, an orthorhombic system, space group P 2$_1$2$_1$2$_1$, Z = 2, a = 10.1348(2) Å, b = 10.2248(2) Å, c = 25.1440(18) Å, V = 2605.6 (2) Å^3, $D_{calcd.}$ = 1.184 g/cm^3, $F(000)$ = 1,008, μ = 0.690 mm^{-1}, 17,654 collected reflections (3.516° $\leq \theta \leq$ 68.228°, $-9 \leq h \leq$ 12, $-9 \leq k \leq$ 12, $-30 \leq l \leq$ 30), 4681 independent reflections (R_{int} = 0.0340), goodness of fit on F^2: S = 1.138, R_1 = 0.0594 and wR_2 = 0.1561 for all 4681 reflections, R_1 = 0.0508 and wR_2 = 0.1350 for 4020 observed reflections [$I > 2\sigma(I)$], refining 308 parameters and 6 restraints, semi-empirical absorption corrections from multi-scans (T_{min} = 0.77, T_{max} = 0.92), and a final electron density between -0.186 and 0.258 e.Å^{-3}.

3.5. Virus-cell-based antiviral assay

Throughout the experiments, Vero (African green monkey kidney) cells and chikungunya virus strain 899 were used (Bourjot et al., 2012). Serial dilutions of extract, fractions or pure compounds, as well as the reference compound chloroquine (positive control), were prepared in an assay medium [MEM] Rega3 (Cat. N°19993013; Invitrogen), 2% FCS (Integro), 5 mL of 200 mM L-glutamine, and 5 mL of 7.5% sodium bicarbonate that was added to empty wells of a 96-well microtiter plate (Falcon, BD) on a liquid handling platform (Freedom EVO200, Tecan). Subsequently, 50 μL of a 4× virus dilution in assay medium were

added, followed by 50 μL of a cell suspension. This suspension, with a cell density of 25,000 cells/50 μL, was prepared from a Vero cell line that was sub-cultured in a cell growth medium (MEM Rega3 supplemented with 10% FCS, 5 mL of L-glutamine and 5 mL of sodium bicarbonate) at a ratio of 1:4 and grown for seven days in 150 cm^2 tissue culture flasks (Techno Plastic Products). The assay plates were returned to the incubator for six to seven days (37 °C, 5% CO$_2$, 95–99% relative humidity), a length of time in which maximal virus-induced cell death or cytopathic effect (CPE) can be observed in untreated, infected controls. Subsequently, the assay medium was aspirated, replaced with 75 μL of a 5% MTS (Promega) solution in a phenol red-free medium and incubated for 1.5 h. Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan); optical densities (OD values) reached 0.6–0.8 for the untreated, uninfected controls. Raw data were converted to a percentage of the controls and the EC$_{50}$ (50% effective concentration or concentration, which is calculated to inhibit virus-induced cell death by 50%) and CC$_{50}$ values (50% anti-metabolic concentration or a concentration that is calculated to inhibit the overall cell metabolism by 50%) were derived from the dose–response curves. All assay conditions producing an antiviral effect exceeding 50% were checked microscopically for minor signs of CPE or adverse effects on the host cell (*i.e.*, altered cell morphology). A compound is only considered to elicit a selective antiviral effect on virus replication when following microscopic quality control, at least at one concentration of compound, and when no CPE nor any adverse effect is observed (image resembling untreated, uninfected cells). Multiple, independent experiments were performed.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.04.023.

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