ORGANIC LETTERS

2008
Vol. 10, No. 7
1397−1400

Chloropupukeananin, the First Chlorinated Pupukeanane Derivative, and Its Precursors from *Pestalotiopsis fici*

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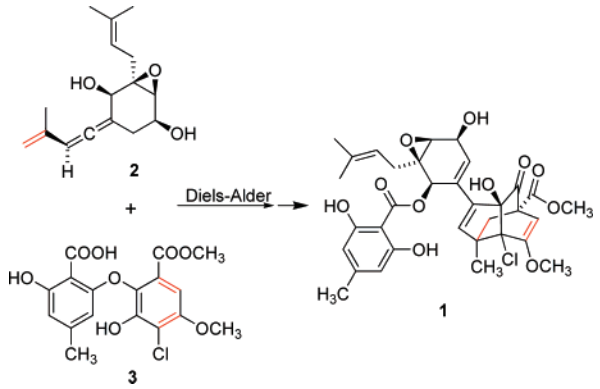
Received January 20, 2008

ABSTRACT



Chloropupukeananin (1), the first pupukeanane chloride with highly functionalized tricyclo-[4.3.1.0^{3,7}]-decane skeleton and its possible biosynthetic precursors iso-A82775C (2) and pestheic acid (3), have been isolated from the plant endophyte *Pestalotiopsis fici*. The structure of 1 was determined by NMR spectroscopy and X-ray crystallography. Biogenetically, 1 could be derived from the Diels–Alder adduct of 2 and 3.

Pupukeananes are a group of sesquiterpenoids possessing the unique tricyclo-[4.3.1.0^{3,7}]-decane skeleton, and they were mainly isolated from marine sponges as isocyanates, thiocyanates, and isothiocyanates. Examples include 2-isocyanopupukeanane and 9-isocyanopupukeanane, the marine invertebrate allomones isolated from the nudibranch *Phyllidia varicosa* and its prey, a sponge *Hymeniacidon* sp.;[1,2] the C-9 epimer of 9-isocyanopupukeanane from the nudibranch *P. bourguini*;[3] 2-thiocyanatopupukeanane from a Palauan sponge of *Axinyssa aplysinoides*;[4] 9-isothiocyanatopupukeanane from the sponge *Axinyssa* sp.[5] In addition, some neopupukeananes have also been discovered from marine sponges, such as 2- and 4-thiocyanatoneopupukeanane,[4,6] and 9-isocyanoneopupukeanane.[7] To our knowledge, the only metabolite from terrestrial sources with the tricyclo-[4.3.1.0^{3,7}]-decane core

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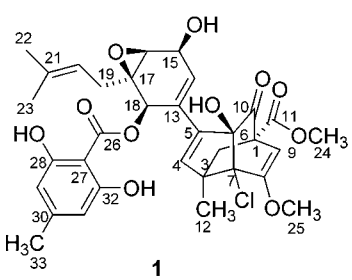
10.1021/ol800136t CCC: $40.75
Published on Web 03/04/2008

carbon skeleton was nemorosonol, a polyisoprenylated pupukeanane isolated from the fruits of *Clusia nemorosa*, and the leaves of a vietnamese *Garcinia bracteata*.[8,9]



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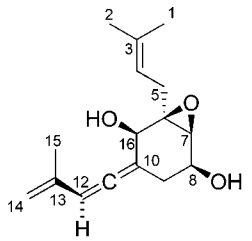

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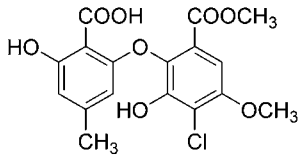

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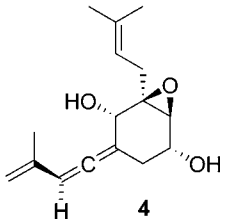

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Many fungal species of the genus *Pestalotiopsis* are saprobes, whereas others are either pathogenic or endophytic on living plants.[10] Chemical investigations of *Pestalotiopsis* spp. have afforded a variety of bioactive natural products.[11−19] During an ongoing search for new bioactive metabolites from plant endophytes, a subculture of an isolate of *P. fici* (W106−1), obtained from branches of an unidentified tree in the suburb of Hangzhou, People's Republic of China, was grown in solid-substrate fermentation culture. Its organic solvent extract displayed an inhibitory effect on HIV-1 replication in C8166 cells. Bioassay-guided fractionation of this extract led to the isolation of a novel pupukeanane chloride, which we named chloropupukeananin (**1**), iso-A82775C (**2**), an isomer of the known fungal metabolite A82775C (**2**),[20] and the known compound pestheic acid (**3**).[21] Details of the isolation, structure elucidation, biogenesis, and biological activity of **1** are reported herein.

1 was obtained as a white powder. Its molecular formula was determined as $C_{33}H_{35}ClO_{11}$(16 degrees of unsaturation) by analysis of its HRESIMS (*m*/*z* 665.1723 $[M + Na]^+$) and NMR data (Table 1). Analysis of the ^1H, ^{13}C, and HMQC

Table 1. NMR Spectroscopic Data for **1** in Acetone-d_6

pos.	δ_H[a] (*J* in Hz)	δ_C[b]	HMBC (H → C#)
1		62.5	
2a	1.56, d (13)	48.7	1, 3, 4, 9, 10, 11, 12
2b	2.67, d (13)		1, 3, 4, 7, 10, 11, 12
3		48.9	
4	6.00, s	138.8	2, 3, 5, 6, 7, 12, 13
5		139.1	
6		86.5	
7		88.5	
8		156.2	
9	5.50, s	92.9	1, 2, 7, 8, 10, 11
10		196.4	
11		169.2	
12	1.01, s	21.9	2, 3, 4, 7
13		125.7	
14	6.35, d (1.0)	133.1	5, 16, 18
15	4.56, ddd (8.0, 3.0, 1.0)	65.3	14
16	3.48, brd (3.0)	61.1	14, 15, 17, 19
17		61.4	
18	6.43, br s	70.9	5, 13, 14, 19, 26
19a	2.40, dd (16, 7.0)	33.0	16, 17, 18, 20, 21
19b	2.48, dd (16, 7.0)		16, 17, 18, 20, 21
20	5.15, t (7.0)	118.2	17, 19, 22, 23
21		136.1	
22	1.53, s	25.7	20, 21, 23
23	1.58, s	18.1	20, 21, 22
24	3.75, s	52.8	11
25	3.66, s	56.6	8
26		170.9	
27		98.5	
28		162.0	
29	6.27, s	109.5	26, 27, 28, 31, 33
30		149.3	
31	6.27, s	109.5	26, 27, 29, 32, 33
32		162.0	
33	2.23, s	21.9	29, 30, 31
OH−6	5.66, s		5, 6, 10
OH−15	4.51, d (8.0)		15
OH−28	9.73, s		27, 28, 29
OH−32	9.73, s		27, 32, 31

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

NMR spectroscopic data of **1** revealed the presence of four exchangeable protons, six methyl groups (two methoxys), two methylenes, three oxymethines, 14 olefinic or aromatic carbons (six of which are protonated), five quaternary carbons (three of which are attached to heteroatoms), and three carbonyl carbons. These data accounted for all ^1H and ^{13}C NMR resonances but one chlorine atom and required chloropupukeananin (**1**) to be hexacyclic. Interpretation of the $^1H-^1H$ COSY NMR data led to the identification of two isolated proton spin-systems corresponding to the C-14−C-

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16 (including OH-15) and C-19−C-20 subunits of structure **1**. In the ^{1}H NMR spectrum of **1**, the signal resonated at 6.27 ppm was integrated for two protons (H-29 and H-31), suggesting the presence of a symmetrical benzene ring. HMBC correlations from H_3-33 to C-29 (δ_C 109.5), C-30, and C-31 (δ_C 109.5) indicated that C-30 was connected to C-29, C-31, and C-33. Correlations of H-29 and H-31 with C-27 (δ_C 98.5), C-28, and C-32 (both C-28 and C-32 were resonated at 162.0 ppm) led to the connection of C-28 to C-27 and C-29, and C-32 to C-27 and C-31, thereby completing the structure of a symmetrical aromatic ring in **1**. Further HMBC correlations from the phenolic protons (δ_H 9.73; OH-28 and OH-32) to C-27, C-28 (C-32), and C-29 (C-31) led to the attachment of the hydroxy groups to C-28 and C-32. An unusual four-bond HMBC correlation from H-29 (H-31) to C-26 indicated that the carboxyl carbon C-26 was directly attached to C-27. On the basis of these data, the structure of a 2,6-dihydroxy-4-methylbenzoate moiety was established.

The structure of the isoprene subunit in **1** was established by HMBC correlations from H_3-22 and H_3-23 to C-20 and C-21. HMBC correlations from H-16 to C-17 and C-19, H-18 to C-19, and from H_2-19 to C-18 indicated that C-16, C-18, and C-19 were all joined to C-17. In turn, correlations from H-18 to C-13 and C-14 and from H-14 to C-18 led to the identification of a cyclohexene moiety with an isoprene unit attached to C-17. An HMBC correlation from H-18 to the carboxyl carbon C-26 established the ester linkage between C-18 and C-26. Other correlations from H_3-12 to C-2, C-3, C-4, and C-7 and from H_2-2 to C-3, C-4, C-7, and C-12 led to the connection of C-2, C-4, and C-12 to either C-3 or C-7. However, the downfield ^{13}C NMR chemical shift for C-7 (δ_C 88.5) and the observation of an HMBC correlation from H-9 to C-7 precluded C-7 from being directly joined to C-2, C-4, and C-12. HMBC correlations from the olefinic proton H-4 to C-5, C-6, and C-7, and from the exchangeable proton at 5.66 ppm (OH-6) to C-5, C-6, and C-10 established the C-3−C-6 partial structure with C-6 directly attached to both a hydroxy group and the ketone carbon C-10 (δ_C 196.4). While those from H_2-2 and H-9 to the quaternary carbon C-1, the ketone carbon C-10, and the carboxyl carbon C-11 led to the connection of C-1 to C-2, C-9, C-10, and C-11. Further correlations from the olefinic proton H-9 to C-7 and C-8 indicated that C-7 was allylic to the C-8/C-9 olefin unit. HMBC correlations from H_3-24 to C-11 and from H_3-25 to C-8 indicated that C-8 and C-11 were each attached to a methoxy group. Key correlations from H-4 to C-13 and from H-14 to C-5 led to the connection of C-5 to C-13. Since all exchangeable protons in **1** were accounted for, the two oxygenated carbons C-16 and C-17 must be attached to the remaining oxygen atom to form an epoxide moiety, and the ^{13}C NMR chemical shifts of C-16 (δ_C 61.1) and C-17 (δ_C 61.4) further supported this assignment. In addition, the chemical shift of C-7 (δ_C 88.5) and the hexacyclic nature of **1** required that C-6 and the chlorine atom be directly attached to C-7 to complete the tricyclo-[4.3.1.0^{3,7}]-decane skeleton. On the basis of these data, the planar structure of chloropupukeananin was established as **1**.

Ultimately, the structure of **1** was confirmed by single-crystal X-ray crystallographic analysis,[22] and a perspective ORTEP plot is shown in Figure 1. Due to the presence of

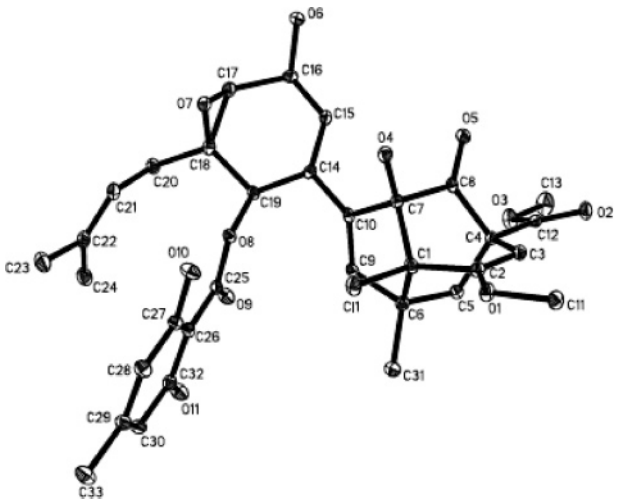

Figure 1. Thermal ellipsoid representation of **1**.

one chlorine atom, the X-ray data also allowed determination of the absolute configuration of all chiral centers in **1** (1*S*, 3*R*, 6*S*, 7*R*, 15*S*, 16*S*, 17*S*, and 18*R*).

2 was assigned the molecular formula $C_{16}H_{22}O_3$ on the basis of its ESIMS and NMR data.[23] Literature search on this formula quickly identified a known precedent, A82775C (**4**), with the same elemental composition as **2**.[20] Detailed comparison of the ^{1}H and ^{13}C NMR data for these two compounds revealed that **2** is a stereoisomer of **4** at C-8 and C-16, and this conclusion was supported by analysis of its NOESY data and by analogy to the absolute configuration established for the cyclohexene moiety in **1** by X-ray data.

1 was tested for *in vitro* activity against HIV-1 replication in C8166 cells,[24] and it showed an inhibitory effect with an IC_{50} value of 14.6 μM (the positive control indinavir sulfate showed an IC_{50} value of 8.18 nM). It also displayed antimicrobial activity against *Staphylococcus aureus* (ATCC 6538), with IC_{50} and MIC values of 21.8 and 97.3 μM, respectively (the positive control AMP showed IC_{50} and MIC values of 1.2 and 3.9 μM at the same concentration).[25−27]

Some terpenoids with the tricyclo-[4.3.1.0^{3,7}]-decane skeleton have been reported previously, but mostly from marine sponges as isocyanates, thiocyanates, and isothiocyanates,[1−7]

(22) Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 674929). Copies of the data can be obtained, free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or email: deposit@ccdc.cam.ac.uk).

(23) Please see Supporting Information.

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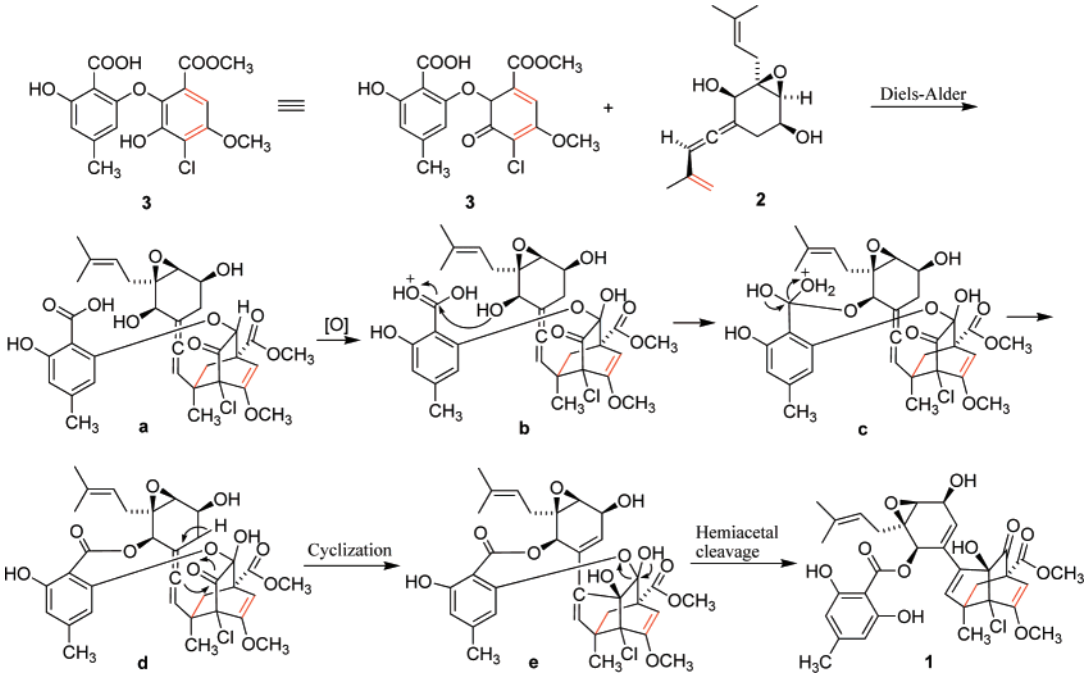
Scheme 1. Proposed Biosynthetic Pathway for **1**



with nemorosonol as the only example from the plant sources.[8,9] Structurally, **1** possesses a unique and highly functionalized tricyclo-[4.3.1.0^{3,7}]-decane skeleton, with a sesquiterpenoid moiety attached to C-5, and a 2,6-dihydroxy-4-methylbenzoic acid unit further connected to the sesquiterpenoid via an ester linkage. **1** is the first chlorinated pupukeanane derivative discovered from any sources, and its tricyclo core skeleton was encountered for the first time for fungal metabolites. **2** was a stereoisomer of the known compound **4**, which was first isolated from an unidentified fungus,[20] whereas **3** was initially discovered from the plant pathogenic fungus *Pestalotiopsis theae* as a plant growth regulator.[21] In our study, these two metabolites were both identified from *P. fici*, and they appeared to be the biosynthetic precursors for chloropupukeananin (**1**), first via Diels−Alder reaction,[28] and then followed by a series of reactions as illustrated in Scheme 1.

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1 is the first secondary metabolite to be reported from the plant endophytic fungus *P. fici*, and the discovery of this structurally unique compound further demonstrated that the plant endophytic fungi could be important sources for bioactive natural products.

Acknowledgment. We thank Dr. Liqiang Chen of the Center for Drug Design, the University of Minnesota for helpful discussions. We gratefully acknowledge generous financial support from the Key Program of the National Hi-Tech Research and Development (Grant 2007AA021506), the Key Project of the Chinese Academy of Sciences (Grant KSCX2-Y-G-013), and the National Natural Science Foundation of China (Grant 30670055).

Supporting Information Available: Experimental procedures, characterizationn data, ^{1}H, and ^{13}C NMR spectra of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL800136T