JOURNAL OF NATURAL PRODUCTS

Article

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Anti-inflammatory 12,20-Epoxypregnane and 11,12-*seco*-Pregnane Glycosides from the Stems of *Hoya kerrii*

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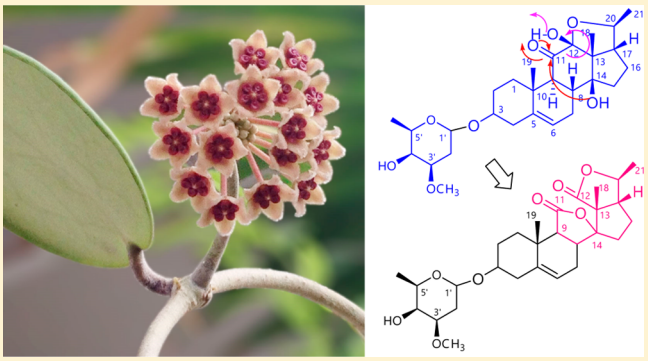
Supporting Information

ABSTRACT: Five 12,20-epoxypregnane glycosides (**1**−**3**, **5**, and **6**) and two 11,12-*seco*-pregnane glycosides (**4** and **7**) with spirodilactone motifs, as well as spirodilactone cleavage products **8** and **9**, were isolated from the stems of *Hoya kerrii*. The relative configurations of the three related skeletons were supported by ROESY experiments and X-ray crystallographic analyses. The isolates were evaluated for their anti-inflammatory activity based on the inhibition of NO production in RAW264.7 cells, and some showed IC_{50} values ranging from 12.6 to 96.5 μM. The most potent compound, **9a**, was also examined for its anti-inflammatory mechanism against mRNA expression and was found to down-regulate mRNA expression of iNOS and COX-2 in a dose-dependent manner.



Hoya kerrii Craib.[1] [syn. *H. obovata* Decne. *var. kerrii* (Craib.) Costantin] of the Apocynaceae family is commonly known in Thailand as "Dang and Tang". This plant is among 53 *Hoya* species found in Thailand.[1] The latex, stems, and leaves of this plant have been reported to possess wound-healing and anti-inflammatory properties. A water decoction of the leaves has been used as an antidiabetic treatment.[2] There have been reports dealing with the chemical constituents of several *Hoya* plants, including *Hoya australis*,[3] *H. carnosa*,[4,5] *H. lacunose*,[6] *H. naumanii*,[7] and *H. parasitica*,[8] that led to the isolation of some 3,4-*seco*-pentacyclic triterpenoids,[3,6−8] pregnanes,[4] pregnane glycosides,[4] androstanes,[8] sesquiterpenoids,[8] phenolic compounds,[8] and oligosaccharides.[5] However, there have been no reports on any phytochemical investigation of *H. kerrii*. The preliminary anti-inflammatory activity assessment of the inhibition of nitric oxide production in RAW264.7 cells indicated that the CH_2Cl_2 extract of the stem was active, showing an IC_{50} value of 57.2 μg/mL. An additional in-house brine shrimp lethality assay indicated that the CH_2Cl_2 extract of the stems at a concentration of 250 ppm caused 100% lethality of *Artemia salina* nauplii. The use of a combination of chromatographic techniques led to the isolation of several new steroids (**1**−**9**) in addition to the seven known compounds lupeol acetate,[9] taraxerol,[10] lupeol,[10] a mixture of sitosterol and stigmasterol, *p*-hydroxybenzaldehyde, scopoletin,[11] and lariciresinol.[12] We report herein the spectroscopic identification of compounds **1**−**9** and their anti-inflammatory activity.

RESULTS AND DISCUSSION

Compound **1** was obtained as a colorless solid that showed an HRESIMS sodium adduct molecular ion at *m*/*z* 513.2828, corresponding to a molecular formula of $C_{28}H_{42}O_7$. The FTIR indicated peaks for the presence of a carbonyl and an olefin function at ν_{max} 1711 and 1661 cm^{-1}, respectively. The ^{13}C NMR spectrum revealed the presence of 28 carbon signals comprising five methyl, seven methylene, 11 methine (including seven oxymethine and one olefinic), and five nonprotonated carbons. Among the nonprotonated carbons was a carbonyl, an oxygenated tertiary carbon, and an olefinic carbon. The ^1H NMR resonance at δ_H 4.49 (dd, J = 9.8 and 1.9 Hz) showed an HMQC cross-peak with a dioxygenated methine carbon at δ_C 98.1, assignable to an anomeric H-1′, together with $^1H-^1H$ COSY cross-peaks between H-2′/H-1′

Received: September 4, 2016

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

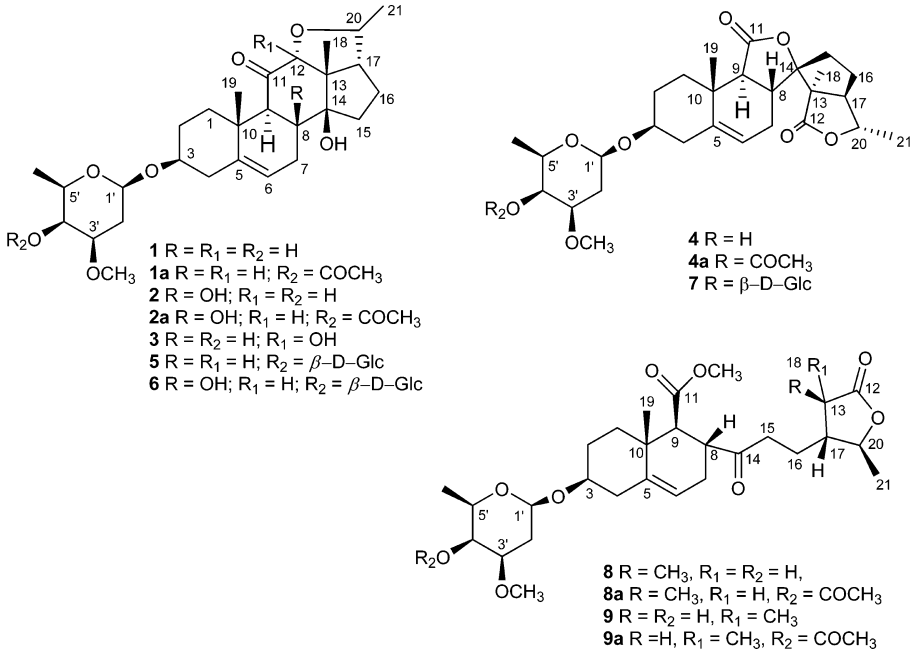


Figure 1. Structures of the isolated compounds and their derivatives.

and H-3′, H-4′/H-3′ and H-5′, and H-5′/H_3-6′. These correlations permitted the establishment of the presence of a 2,6-dideoxypyranose unit in **1** (Table 1). The coupling constants of the oxymethine protons and the HMBC cross-peak of the OMe singlet at δ_H 3.37 with C-3′ (δ_C 78.0) indicated the presence of a *β*-diginopyranosyl ring.[13] The remaining 21 carbon resonances of the aglycone with two methyl groups each attached to tertiary carbons and thus resonating in the ^{1}H NMR spectrum as two singlets at δ_H 1.35 and 1.01 showed that the aglycone contained a pregnane moiety. The olefinic function, as observed by a ^{1}H NMR resonance at δ_H 5.45 d (J = 5.4 Hz) and ^{13}C NMR resonances at δ_C 140.9 (C) and 121.7 (CH), was assigned at C-5(6), as evident from the HMBC cross-peaks between H_3-19 (δ_H 1.01)/C-1 (δ_C 37.4), C-5, and C-10 (δ_C 39.4) as well as H-3/C-1 and C-5 (Figure S45, Supporting Information). The methyl singlet at δ_H 1.35 assigned to H_3-18 showed HMBC cross-peaks with carbon resonances at δ_C 82.2 (C-14), 91.5 (C-12), and 58.4 (C-17). The presence of a ^{1}H NMR singlet at δ_H 3.88, showing an HMQC cross-peak with C-12, HMBC cross-peaks not only with C-14, C-17, and C-18 (δ_C 22.7) but also with the carbonyl and the oxymethine carbons at δ_C 211.0 and 82.3 (assigned to C-20), respectively, required the presence of a carbonyl group at C-11 and an ether linkage between C-12 and C-20. On the basis of these data, the aglycone in **1** was defined as 3,14-dihydroxy-12,20-epoxypregn-5-en-11-one. The connectivity between the oxygen atom at C-3 and C-1′ of the diginopyranose unit was evident from the HMBC cross-peaks between H-3/C-1′ and H-1′/C-3. The *O*-acetyl derivative **1a** was obtained after reacting **1** with Ac_2O−pyridine, and its ROESY spectrum showed cross-peaks between H_3-18/H-8, H-12, and H-17, while H_3-19 showed cross-peaks with H-8 and H-12. The anomeric H-1′ showed NOE correlations with H-3, H-3′, and H-5′ (Figure 2a). A single-crystal X-ray crystallographic analysis of **1** provided the ORTEP plot shown in Figure 2b. The absolute configuration of the sugar was assigned after acid hydrolysis of **1** with 0.05 M HCl at 60 °C for 4 h to give the aglycone 12*α*,20*α*-epoxy-14*β*-hydroxy-pregn-5-en-11-one and D-diginose, $[\alpha]^{26}_D$ +68 (*c* 0.1, H_2O), lit.[13,14a] +59.6 (*c* 1.48, H_2O). The structure of compound **1** was thus elucidated as 12*α*,20*α*-epoxy-14*β*-hydroxypregn-5-en-11-one 3-*O*-*β*-D-diginopyranoside. Related rare steroid glycosides having ether linkages between C-12 and C-20 but with an additional C-15 carbonyl group and no 14-hydroxy function, e.g., 12*α*,20*α*-epoxypregn-5-en-11,15-dione 3-*O*-*β*-D-diginopyranoside (digitinin), have been isolated from *Digitalis ciliate* and *D. purpurea*.[14]

Compound **2** was obtained as a colorless solid with a molecular formula of $C_{28}H_{42}O_8$ {HRESIMS [M + Na]$^+$ *m*/*z* 529.2759 (calcd for $C_{28}H_{42}O_8Na$, 529.2771)}, thus implying that **2** has one more oxygen atom than **1**. The ^{1}H and ^{13}C NMR resonances of **2** showed close resemblance to those of **1** (Table 1), indicating the presence of diginopyranosyl and 12*α*,20*α*-epoxy-14*β*-hydroxypregn-5-en-11-one moieties, except that H-12 resonated at δ_H 4.18, i.e., deshielded relative to **1**. Since the ^{1}H NMR resonance for H-9 (δ_H 2.20) was a singlet, instead of a doublet as in **1**, and both H-9 and H-6 (δ_H 5.38) showed HMBC cross-peaks with the same oxygenated tertiary carbon at δ_C 76.7, the presence of the hydroxy group at C-8 was indicated. On the basis of the acid hydrolysis of **1**, which provided D-diginose, the structure of compound **2** was therefore elucidated as 12*α*,20*α*-epoxy-8*β*,14*β*-dihydroxypregn-5-en-11-one 3-*O*-*β*-D-diginopyranoside. The 4′-*O*-acetyl derivative **2a** was obtained after reacting **2** with Ac_2O in pyridine (Table S1, Supporting Information).

Compound **3**, a colorless solid, had the same molecular mass as **2** based on HRESIMS. The ^{1}H and ^{13}C NMR spectra also exhibited similar patterns of resonances to those observed in **2**, except that the ^{1}H NMR singlet at ca. δ_H 4.18 and the ^{13}C NMR resonance at δ_C 90.6, assigned to H-12 and C-12 in **2**, were absent (Table 1). The important HMBC cross-peaks of H-9 (δ_H 2.28) and H-17 (δ_H 2.07) with a dioxygenated carbon at δ_C 102.7 led to the placement of a hydroxy group at C-12. Acid hydrolysis of **1** afforded D-diginose; thus the structure of compound **3** was assigned as 12*α*,20*α*-epoxy-12*β*,14*β*-dihydroxypregn-5-en-11-one 3-*O*-*β*-D-diginopyranoside.

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. ^{1}H NMR Spectroscopic Data (400 MHz) of 1−4 (in $CDCl_3$, J in Hz)

	1		2		3		4	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	2.35,[a] 1.33	37.4, CH_2	2.36, 1.24	38.4, CH_2	2.34, 1.20	37.2, CH_2	2.40, 1.22	36.5, CH_2
2	1.99,[b] 1.55	29.1, CH_2	1.95,[d] 1.63	28.7, CH_2	1.92, 1.58	29.2, CH_2	1.98, 1.21	29.0, CH_2
3	3.55, dddd (11.4, 6.6, 4.6, 1.2)	77.7, CH	3.57, dddd (11.2, 6.2, 5.0, 1.2)	77.9, CH	3.57[g]	77.2, CH	3.56	77.5, CH
4	2.33,[a] 2.15, brt (11.4)	38.3, CH_2	2.41, 1.26	39.9, CH_2	2.23, 1.25	38.3, CH_2	2.34, 2.18	38.1, CH_2
5		140.9, C		142.5, C		140.6, C		141.0, C
6	5.45, d (5.4)	121.7, CH	5.38, d (5.6)	117.8, CH	5.38, brs	120.5, CH	5.32, brs	120.6, CH
7	2.27, 1.76, brd (14.8)	28.1, CH_2	2.37, 2.17[e]	35.3, CH_2	2.31, 2.05	28.1, CH_2	2.58, 2.12	25.4, CH_2
8	2.34[a]	36.6, CH		76.7, C	2.28[h]	37.3, CH	2.55	43.1, CH
9	1.97, d (12.4)[b]	59.6, CH	2.20, s[e]	59.4, CH	2.28[h]	55.0, CH	2.54	51.5, CH
10		39.4, C		39.7, C		38.7, C		39.7, C
11		211.0, C		211.0, C		207.1, C		175.1, C
12	3.88, s	91.5, CH	4.18, s	90.6, CH		102.7, C		179.1, C
13		63.2, C		62.6, C		63.1, C		56.8, C
14		82.2, C		83.3, C		82.9, C		93.9, C
15	1.92,[c] 1.52	32.4, CH_2	2.37, 1.74	34.6, CH_2	1.70	33.5, CH_2	2.12	39.7, CH_2
16	1.92,[c] 1.29	25.9, CH_2	1.85, 1.24	25.8, CH_2	1.42, brdd (10.6, 4.3)	28.3, CH_2	2.32, 1.61	27.0, CH_2
17	1.83, brt (8.1)	58.4, CH	1.81, brt (7.7)	60.6, CH	2.07	59.2, CH	2.33	56.6, CH
18	1.35, s	22.7, CH_3	1.52, s	25.4, CH_3	1.21, s	16.2, CH_3	1.35, s	23.0, CH_3
19	1.01, s	19.9, CH_3	1.15, s	18.8, CH_3	1.15	19.9, CH_3	1.08, s	18.3, CH_3
20	3.47, brq (5.9)	82.3, CH	3.45, quint (6.3)[f]	81.2, CH	3.60, brq (6.1)[g]	81.9, CH	4.25, quint (6.2)	80.0, CH
21	1.31, d (5.9)	20.2, CH_3	1.30, d (6.2)	20.3, CH_3	1.34, d (6.1)[i]	22.5, CH_3	1.40, d (6.2)	21.7, CH_3
1′	4.49, dd (9.8, 1.9)	98.1, CH	4.50, dd (9.8, 2.1)	98.1, CH	4.50, brd (9.6)	97.9, CH	4.48, d (9.7)	98.2, CH
2′	1.95,[b] 1.68	32.0, CH_2	1.96,[d] 1.65	31.9, CH_2	1.94, brd (12.5), 1.65	32.0, CH_2	1.92, 1.64	32.0, CH_2
3′	3.31, ddd (12.0, 4.7, 3.1)	78.0, CH	3.31, ddd (12.0, 4.8, 3.1)	77.8, CH	3.31, ddd (11.9, 4.5, 2.2)	78.0, CH	3.30, ddd (11.9, 4.4, 2.2)	78.0, CH
4′	3.66, brs	67.1, CH	3.66, brd (3.1)	67.0, CH	3.67, brs	67.1, CH	3.66, brs	67.1, CH
5′	3.42, brq (6.4)	70.5, CH	3.43, brq (6.7)[f]	70.5, C	3.43, brq (6.4)	70.4, CH	3.42, brq (6.3)	70.4, CH
6′	1.31, d (6.4)	16.8, CH_3	1.31, d (6.7)	16.8, CH_3	1.33, d (6.4)[i]	16.8, CH_3	1.31, d (6.3)	16.8, CH_3
3′-OCH_3	3.37, s	55.7, CH_3	3.37, s	55.7, CH_3	3.41, s	55.7, CH_3	3.36, s	55.7, CH_3
4′-$COCH_3$								

[a−i]Overlapped signals.

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

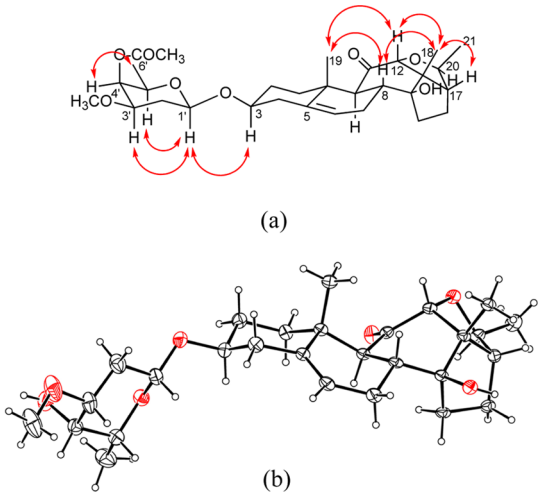
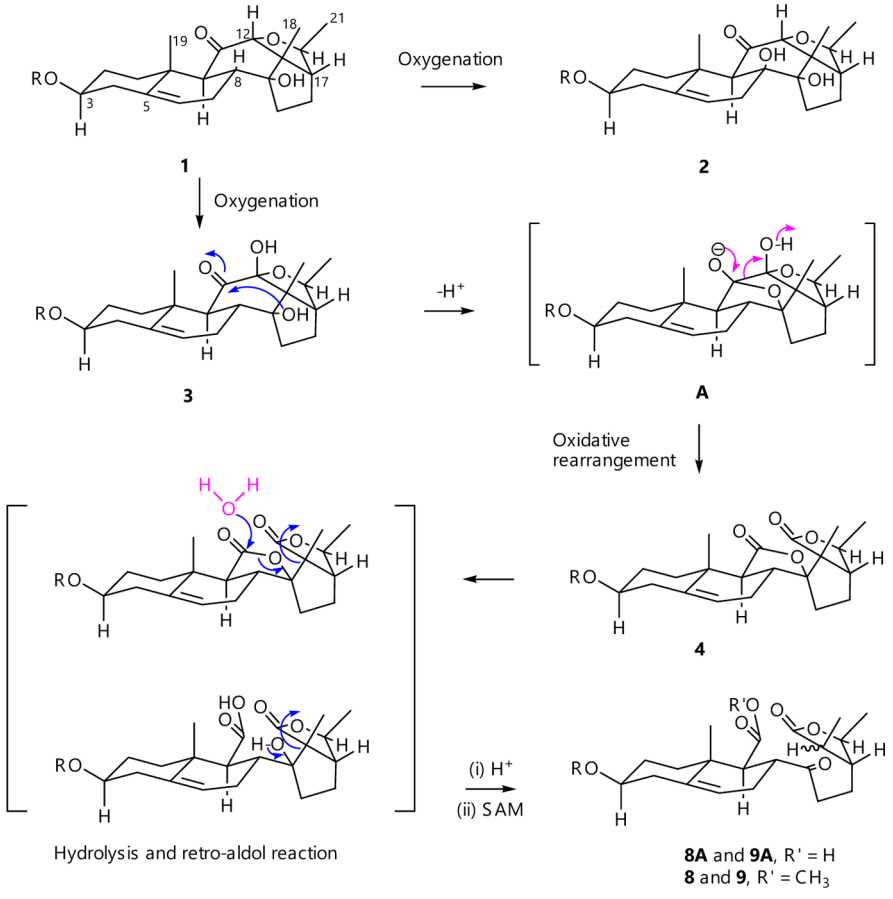


Figure 2. (a) Key NOE correlations of **1a** and (b) an ORTEP plot of **1**.

Compound **4** was isolated as a colorless solid with the molecular formula of $C_{28}H_{40}O_8$ based on its HRESIMS spectrum, showing an $[M + Na]^+$ ion at m/z 527.2625 (calcd for $C_{28}H_{40}O_8Na$, 527.2615). The ^{13}C NMR spectrum showed 28 carbons comprising five methyl, seven methylene, 10 methine, and six nonprotonated carbons including two deshielded carbonyl carbons (δ_C 175.1 and 179.1), one olefinic carbon (δ_C 141.0), one oxygenated tertiary carbon (δ_C 93.9), and two quaternary carbons. The ^1H NMR resonances of a diginopyranosyl moiety [δ_H 4.48 d (J = 9.7 Hz, H-1′), 1.31 (d, J = 6.3 Hz, H_3-6′)] were also apparent (Table 1). The other methyl doublet at δ_H 1.40 (J = 6.2 Hz), assigned to H_3-21, showed a $^1H-^1H$ COSY cross-peak with a deshielded quintet at δ_H 4.25 (H-20). This deshielded signal compared with those of C-20 in **1**−**3** suggested the attachment of an *O*-acyl group to C-20. The HMBC cross-peaks between H_3-18/C-17, the less-shielded C-14 (δ_C 93.9), and a carbonyl carbon (δ_C 179.1, assigned to C-12), as well as an H-20/C-12 cross-peak (Figure S45, Supporting Information), required a lactone linkage between C-20 and C-13. The other important HMBC cross-peaks of both H-8 (δ_H 2.55) and H-9 (δ_H 2.54) with C-14 and a carbonyl carbon at δ_C 175.1 required another lactone moiety between C-14 and C-9. Compound **4** could therefore be identified as an 11,12-*seco*-pregnane derivative possessing a spirodilactone motif (Scheme 1, postulated transformation). Acetylation of **4** using Ac_2O−pyridine gave **4a**. The ROESY spectrum of **4a** showed cross-peaks between H-8/H_3-19, H-17/H_3-18 and H_3-21, and H_3-18/H_3-21, as well as cross-peaks between H-1′/H-3, H-3′, and H-5′. The X-ray crystallographic structure of **4a**, as illustrated in Figure 3a and b, defined the relative configurations of the stereogenic centers in **4**. On the basis of the acid hydrolysis of **1**, which gave D-diginose, the structure of compound **4** was proposed as kerriipregnane A 3-*O*-*β*-D-diginopyranoside.

Compound **5** was obtained as a colorless solid. The HRESIMS revealed a molecular formula of $C_{34}H_{52}O_{12}$. The ^1H and ^{13}C NMR spectra established resonances of the diginopyranosyl and the 12*α*,20*α*-epoxy-14*β*-hydroxypregn-5-

Scheme 1. Postulated Biosynthesis Transformations of 1−3, 4, and 8/9



DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

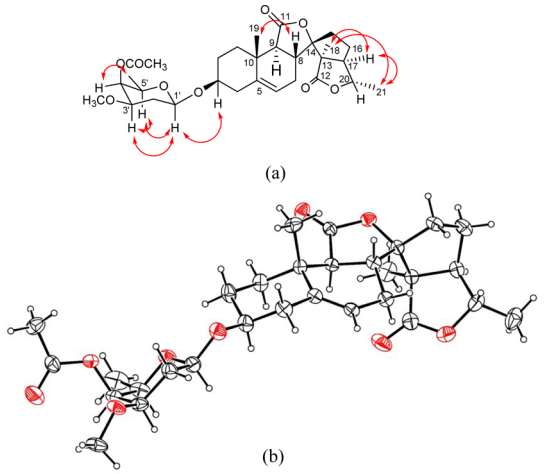


Figure 3. (a) Key NOE correlations and (b) an ORTEP plot of **4a**.

en-11-one skeletons as found in **1** (Table 2). The presence of a glucopyranosyl ring was indicated by the ^{1}H and ^{13}C NMR resonances at δ_H 5.18 d (J = 7.6 Hz, H-1″), δ_C 104.6 (CH, C-1″), δ_H 4.57 dd (J = 11.4 and 1.3 Hz, H-6″a), 4.33 dd (J = 11.4 and 5.9 Hz, H-6″b), and δ_C 63.3 (CH_2, C-6″). The HMBC cross-peaks between H-4′/C-1″ and H-1″/C-4′ indicated the attachment of C-1″ of the glucopyranosyl ring to the oxygen atom at C-4′. Upon acid hydrolysis of **5** with 0.05 M HCl at 60 °C for 6 h and subsequent chromatographic purification, the same aglycone as that of **1**, together with D-diginose, β-D-glucopyranosyl-(1→4)-D-diginopyranose, and D-glucose, was obtained. Compound **5** was therefore identified as 12α,20α-epoxy-14β-hydroxypregn-5-en-11-one 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside.

Compound **6** was isolated as a colorless solid with the molecular formula of $C_{34}H_{52}O_{13}$ based on HRESIMS. The ^{1}H and ^{13}C NMR spectra (Table 2) showed sets of resonances of the diginopyranosyl and the 12α,20α-epoxy-8β,14β-dihydroxypregn-5-en-11-one units as observed in **2**, but with extra ^{1}H and ^{13}C NMR resonances of a glucopyranosyl unit. The HMBC cross-peak between H-1″/C-4′ confirmed the connectivity of C-4′-O to C-1″. On the basis of the acid hydrolysis of **5**, which provided D-diginose and D-glucose, the structure of compound **6** was thus assigned as 12α,20α-epoxy-8β,14β-dihydroxypregn-5-en-11-one 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside.

Compound **7** was isolated as a colorless solid with the molecular formula $C_{34}H_{50}O_{13}$ based on HRESIMS. The ^{1}H and ^{13}C NMR spectra showed similar sets of resonances to those found in **4** (Table 2) but with the presence of additional signals of a glucopyranosyl moiety. The HMBC cross-peak between H-1″ and C-4′ indicated connectivity between C-1″ and the oxygen atom at C-4′ and led to the structural assignment of compound **7** as kerriipregnane A 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside.

Compound **8** was obtained as a colorless sticky liquid with a molecular formula of $C_{29}H_{44}O_9$ based on HRESIMS. The ^{13}C NMR spectrum showed 29 carbon resonances comprising six methyl, seven methylene, 11 methine, and five nonprotonated carbons, among which there were three carbonyl (δ_C 212.4, 178.4, and 174.3), one olefinic (δ_C 140.3), and one quaternary carbon. The ^{1}H NMR spectrum showed resonances for a diginopyranosyl moiety, H-6 (δ_H 5.40) and H-20 (δ_H 4.09, dq, J = 9.1 and 6.1 Hz), similar to those found in **4** but with three methyl doublets [δ_H 1.18 (d, J = 7.6 Hz), 1.42 (d, J = 6.1 Hz, H_3-21), and 1.34 (d, J = 6.5 Hz, H_3-6′)], instead of two as encountered in **4**, together with two methoxy singlets at δ_H 3.65 and 3.39 (OCH_3-3′) (Table 3). The ^{1}H,^{1}H−COSY spectrum exhibited connectivities between the less-shielded H-20/H_3-21 and H-17 (δ_H 1.65), H-17/H-13 (δ_H 2.28) and H-16, and H-13/H_3-18 (δ_H 1.28). The key HMBC cross-peaks between H-17/C-12 (δ_C 178.4), C-13, C-18, C-20, and C-21 revealed a lactone linkage between C-20 and C-13 (Supporting Information). The placements of a carbonyl group at C-14 and an ester carbonyl at C-11 were based on the HMBC cross-peaks between H-8/C-7, C-9, C-10, and C-14 and H-9/C-7, C-10, C-11 (δ_C 174.3), and C-14, respectively. The methoxy singlet at δ_H 3.65 showing an HMBC cross-peak with C-11 was therefore assigned to OCH_3-11. Owing to the overlap of relevant ^{1}H NMR resonances, important NOE correlations could not be unambiguously specified from the ROESY spectrum of either **8** or its acetate **8a**. However, the relative configurations of the stereogenic centers in **8** were obtained from the X-ray structure of **8a**, as shown in Figure 4. On the basis of the acid hydrolysis of **1**, affording D-diginose, the structure of compound **8** was defined as kerriipregnane B 3-O-β-D-diginopyranoside.

Compound **9** was obtained as a sticky liquid, separable from **8** by HPLC purification and having the same molecular formula as that of **8** (HRESIMS $[M + Na]^+$ m/z 559.2881, calcd for $C_{29}H_{44}O_9Na$, 559.2871). The ^{1}H and ^{13}C NMR resonances were similar to those of **8** except that the H-20 resonance was deshielded [δ_H 4.27 quint (J = 6.3 Hz)] and the H_3-18 resonance was shielded [δ_H 1.18 d (J = 7.6 Hz)] (Table 3). On the basis of the postulated transformation route (Scheme 1) and the ROESY spectra of **9** and its O-acetyl derivative **9a**, which exhibited NOE cross-peaks between H-8/H_3-19, H-17/H-13 and H_3-21, H-13/H_3-21, and H-1′/H-3, H-3′, and H-5′ (Figure 5), compound **9** was thus concluded to be the C-13 epimer of **8** and named 13-*epi*-kerriipregnane B 3-O-β-D-diginopyranoside.

We propose that compounds **1**−**3** are inter-related by an oxygenation reaction (Scheme 1). The formation of **4** was proposed from **3** by a nucleophilic attack of the OH-14 group at the C-11 carbonyl function, leading to intermediate **A**. After oxidative rearrangement, resulting in cleavage of the bond between C-11 and C-12, and some biological oxidant (presumably $NADP^+$) accepting a hydride ion, a 11,12-*seco*-pregnane derivative with a spirodilactone motif, **4**, was established. Hydrolysis of **4** followed by a retro-aldol reaction, which could be by either a one- or two-step reaction, could lead to the formation of either **8A** or **9A** depending on which face the protonation occurred. Subsequent methylation, most probably with *S*-adenosylmethionine, provided **8** and **9**. It is, however, noteworthy that compound **4** remained intact after stirring **4** (3 mg) with silica gel (50 mg) in MeOH (5 mL) at room temperature for 5 days.

Table 4 shows the anti-inflammatory activities of selected compounds based on their inhibition of nitric oxide (NO) production in RAW264.7 cells.[16] Among the test compounds, compound **9a** showed the most potent inhibitory activity, with an IC_{50} value of 12.6 μM, which is comparable to that of the positive control, caffeic acid phenethyl ester, CAPE (IC_{50} = 9.3 μM), while compound **8a** exhibited an IC_{50} value of 31.6 μM, which is more potent than aspirin, ibuprofen, indomethacin, and L-nitroarginine (L-NA). Compound **9a** was also examined for its anti-inflammatory mechanism against mRNA expression.

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 2. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Spectroscopic Data of 5−7 (in Pyridine-d_5)

	5		6		7	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	2.61,[a] 1.51	38.2, CH_2	2.71,[e] 1.45	40.4, CH_2	2.55,[h] 1.24	36.4,[j] CH_2
2	2.14, 1.67	33.4, CH_2	2.10, 1.78	29.9, CH_2	2.17,[i] 1.68	29.2, CH_2
3	3.86, dddd (10.9, 10.9, 6.5, 4.6)	77.7, CH	3.90[f]	78.1, CH	3.68, dddd (10.8, 10.8, 6.6, 4.1)	76.5, CH
4	2.61,[a] 2.20[b]	38.9, CH_2	2.59, brdd (11.5, 2.4), 2.32	39.2, CH_2	2.47, 2.27[i]	37.9, CH_2
5		141.0, C		140.9, C		140.6, C
6	5.5, brs	123.1, CH	5.4, brs	119.5, CH	5.28, brs	120.6, CH
7	2.68, 2.27	29.2, CH_2	2.74,[e] 2.38	36.0, CH_2	2.73, 2.15	25.0, CH_2
8	2.24[b]	37.3, CH		77.3, C	2.58[h]	43.3, CH
9	2.21,[b] d (13.7)	60.3, CH	2.44, brs	60.6, CH	2.87, d (14.2)	51.1, CH
10		39.9, C		40.6, C		36.4,[j] C
11		211.9, C		212.9, C		179.2, C
12	4.18, s	92.3, CH	4.58, s	91.8, CH		174.8, C
13		64.2, C		63.4, C		56.2, C
14		81.7, C		85.3, C		93.2, C
15	1.99	30.1, CH_2	1.95	36.4, CH_2	2.66, 2.08[i]	39.5, CH_2
16	1.29	26.7, CH	1.23	26.7, CH_2	2.10,[i] 1.48	26.2, CH_2
17	1.94, dt (13.0, 6.1)	59.4, CH	1.88 [8]	61.4, CH	2.13	56.4, CH
18	1.69, s	24.1, CH_3	1.85, s	27.2, CH_3	1.35, s	23.1, CH_3
19	0.97, s	20.1, CH_3	1.36, s	18.6, CH_3	1.07, s	17.8, CH_3
20	3.69, quint (6.1)	82.7, CH	3.62, quint (6.2)	81.6, CH	4.22, quint (6.4)[k]	79.4, CH
21	1.33, d (6.0)	20.7, CH_3	1.28, d (5.9)	21.0, CH_3	1.21, d (6.2)	20.9, CH_3
1′	4.81, brd (9.4)	98.9, CH	4.78, brd (9.4)	98.9, CH	4.69, brd (9.6)	98.3, CH
2′	2.41, 2.15	32.9, CH_2	2.31, 2.08, brt (9.4)	33.5, CH_2	2.32, 2.08[i]	32.6, CH_2
3′	3.53, ddd (10.7, 3.9, 2.4)	80.3, CH	3.48, brdt (11.5, 3.3)	80.4, CH	3.41, dt (11.0, 3.2)	79.6, CH
4′	4.27, brs[c]	73.4, CH	4.23, brs[g]	73.4, CH	3.59, brs	73.0, CH
5′	3.60[m]	71.0, CH	3.55, brq (6.3)	71.1, CH	3.52, q (6.2)	70.3, CH
6′	1.54, d (6.2)	18.1, CH_3	1.49, d (6.3)	18.2, CH_3	1.48, d (6.3)	17.4, CH_3
3′-OCH_3	3.43, s	56.4, CH_3	3.37, s	56.5, CH_3	3.33, s	55.6, CH_3
1″	5.18, d (7.6)	104.6, CH	5.11, d (7.7)	104.6, CH	5.13, d (7.7)	104.3, CH
2″	3.97[d]	78.4, CH[n]	3.87[f]	78.4, CH	3.90[j]	75.5,[o] CH
3″	4.24,[c] obs t (8.9)	78.7, CH[n]	4.18, t (8.9)	78.8, CH	4.14, t (9.1)[k]	71.4, CH
4″	4.15, obs t (8.9)	72.1, CH	4.08, t (9.1)	72.2, CH	4.12, t (9.1)[k]	78.1, CH
5″	3.99[d]	76.1, CH	3.94[f]	76.2, CH	3.87[j]	77.8,[o] CH
6″	4.57, dd (11.4, 1.3), 4.33, dd (11.4, 5.9)	63.3, CH_2	4.51, brd (11.5), 4.26, dd (11.5, 6.0)[g] 5.69, brs	63.3, CH_2	4.53, brd (10.6), 4.32, dd (10.6, 5.2)	62.7, CH_2

[a−k]Overlapped signals. [m]Obscured by solvent signal. [n,o]Interchangeable signals.

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 3. ^{1}H (400 MHz) and ^{13}C NMR Spectroscopic Data (100 MHz) of 8and 9 and ^{1}H NMR Spectroscopic Data of 8a and 9a (in $CDCl_3$)

	8		9		8a	9a
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_H (J in Hz)
1	2.01[a]	37.7, CH_2	2.02[e]	37.7, CH_2	1.85	1.89
2	2.02,[a] 1.59	29.7, CH_2	2.03,[e] 1.57	29.3, CH_2	2.03, 1.63	2.03, 1.63
3	3.59, dddd (11.2, 10.9, 6.3, 4.0)	77.3, CH	3.59, dddd (11.2, 10.6, 4.6, 4.0)	76.5, CH	3.57[h]	3.57[k]
4	2.38, brdd (13.5, 4.0), 2.23[b]	38.4, CH_2	2.40, dd (13.1. 3.2), 2.21[f]	38.5, CH_2	2.40, dd (13.0, 3.5), 2.24	2.40, dd (13.5, 3.3), 2.24
5		140.3, C		140.3, C		
6	5.40, brs	119.4, CH	5.40, brs	119.5, CH	5.39, brs	5.39, d (2.9)
7	2.27,[b] 1.88	28.7, CH_2	2.21,[f] 1.85	28.7, CH_2	2.26, 1.85	2.23, 1.85
8	3.10, dt (11.8, 5.2)	45.3, CH	3.09, dd (11.7, 5.3)	45.2, CH	3.09, dt (11.7, 4.9)	3.08, dt (11.5, 5.2)
9	2.64,[c] d (11.8)	53.8, CH	2.65, d (11.7)	53.9, CH	2.66, d (11.7)[i]	2.64, d (11.5)[j]
10		37.1, C		37.1, C		
11		174.3, C		174.3, C		
12		178.4, C		178.5, C		
13	2.28, quint (7.1)[b]	41.9, C	2.73, quint (7.7)	37.5, CH	2.29	2.72, quint (7.8)
14		212.4, C		212.6, C		
15	2.67, ddd (17.9, 7.3, 6.9)[c]	39.3, CH_2	2.59, ddd (18.1, 7.1, 5.5)	39.6, CH_2	2.63[i]	2.57[j]
16	1.76[d]	24.6, CH_2	1.70,[g] 1.59	20.4, CH_2	1.76	1.73, 1.60
17	1.65	50.4, CH	2.06	45.3, CH	1.68	2.03
18	1.28, d (7.1)	14.9, CH_3	1.18, d (7.6)	10.3, CH_3	1.26, d (7.1)	1.17, d (7.8)
19	1.01, s	20.3, CH_3	1.00, s	20.3, CH_3	1.00, s	0.99, s
20	4.09, dq (9.1, 6.1)	79.7, CH	4.27, quint (6.3)	79.2, CH	4.07, dq (8.9, 6.1)	4.26, quint (6.3)
21	1.42, d (6.1)	20.0, CH_3	1.39, d (6.3)	19.5, CH_3	1.41, d (6.1)	1.38, d (6.3)
1′	4.51, dd (9.7, 1.9)	98.2, CH	4.52, dd (9.7, 1.6)	98.2, CH	4.55, brd (8.3)	4.55, dd (9.7, 1.4)
2′	1.96, brd (12.4, 4.7), 1.69[d]	31.9, CH_2	1.94, dd (14.1, 3.5), 1.65[g]	32.0, CH_2	1.91, dd (12.1, 3.1), 1.75	1.93, dd (12.2, 3.4), 1.79
3′	3.33, ddd (11.9, 4.7, 3.1)	77.9, CH	3.33, ddd (11.7, 4.7, 3.5)	77.9, CH	3.36[j]	3.36[l]
4′	3.69, brs	67.0, CH	3.69, brs	67.0, CH	5.16, brs	5.16, d (1.9)
5′	3.44, brq (6.5)	70.4, CH	3.44, brq (6.4)	70.4, CH	3.53, brq (6.5)[h]	3.54, brq (6.5)[k]
6′	1.34, d (6.5)	16.8, CH_3	1.34, d (6.4)	16.8, CH_3	1.19, d (6.5)	1.19, d (6.5)
3′-OCH_3	3.39, s	55.7, CH_3	3.39, s	55.7, CH_3	3.33, s[j]	3.33, s[l]
$COOCH_3$-11	3.65, s	51.6, CH_3	3.64, s	51.5, CH_3	3.63, s	3.63, s
$COCH_3$-4′					2.13, s	2.13, s

[a−l]Overlapped signal.

DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX

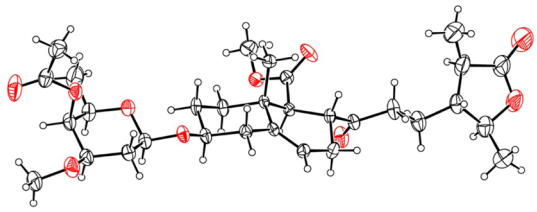


Figure 4. ORTEP plot of **8a**.

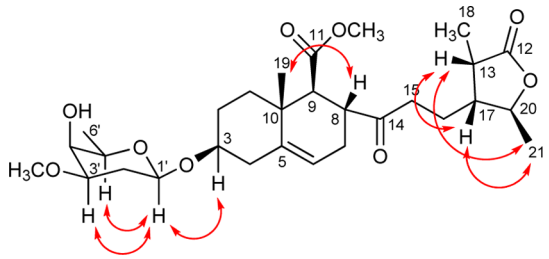


Figure 5. Key NOE correlations of **9**.

Table 4. Anti-inflammatory Activity Based on the Inhibition of Nitric Oxide Production in RAW264.7 Cells[a]

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	96.5[b]	**1a**	91.2
2	62.5	**2a**	87.8
3	84.0	**4**	93.8
4a	60.4	**5**	>100
6	>100	**8**	87.3[c]
8a	31.6	**9**	66.1
9a	12.6[b]	aspirin[d]	43.2
ibuprofen[d]	54.5	indomethacin[d]	46.5
CAPE[d]	9.3	L-NA[d]	37.7

[a]Each value represents mean ± SEM of four determinations. [b]Cytotoxic effect was observed at ≥100 μM (by the MTT assay). [c]Cytotoxic effect was observed at ≥10 μM. [d]Positive control compounds, caffeic acid phenethyl ester (CAPE), L-nitroarginine (L-NA).

The compound was found to down-regulate mRNA expression of iNOS and COX-2 in a dose-dependent manner (Figure 6).

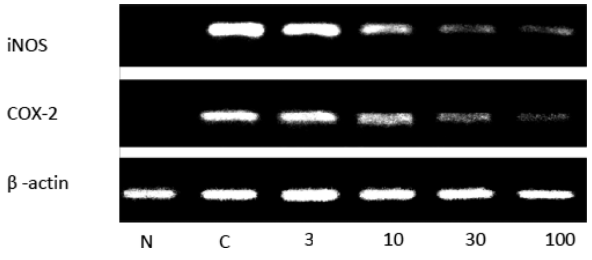


Figure 6. Effect of **9a** at various concentrations (3, 10, 30, 100 μM) on the mRNA expression of iNOS and COX-2 using RAW264.7 cells [N = LPS (−), sample (−); C = LPS (+), sample (−); 3−100 μM = LPS (+), sample (+)].

In summary, five rare 12,20-epoxypregnane glycosides and the first two 11,12-*seco*-pregnane glycosides with a spirodilactone motif, as well as the spirodilactone cleavage products, were isolated from the stems of *H. kerrii*. Their structures were elucidated on the basis of extensive spectroscopic and X-ray crystallographic analyses. The absolute configurations of the sugar units were proven by acid hydrolysis of some selected glycosides. Some compounds showed potent to moderate anti-inflammatory activity.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using an Electrothermal melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP 1020 polarimeter. The IR spectra were obtained on a PerkinElmer 1760x FT-IR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE 400 MHz spectrometer. Chemical shifts are referenced to the residual solvent signals ($CDCl_3$: δ_H 7.24 and δ_C 77.0). HRESIMS was recorded on a Bruker Daltonics microTOF mass spectrometer. HPLC separation was performed using a Merck LiChrospher 100 RP-18 (5 μm, 250 × 4.0 mm) column with a TSP SpectraSYSTEM P2000 pump and a TSP SpectraSYSTEM UV2000 detector.

Plant Material. The plant, *Hoya kerrii* (Apocynaceae), was collected from Padaeng Subdistrict, Chattrakan District, Pitsanuloke Province, on May 12, 2009. The plant was identified by Associate Professor Dr. Nijsiri Ruangrangsi, Faculty of Pharmacy, Rangsit University, Pathumthani. A voucher specimen (SSHK-2009) is maintained at the Chemistry Department, Ramkhamhaeng University.

Extraction and Isolation. Fresh stem (5.2 kg) of *H. kerrii* was cut into small pieces, ground, and soaked in MeOH (24 L) at room temperature for 30 d. The concentrated extract, obtained as a greenish, waxy residue after removal of solvent under reduced pressure, was partitioned successively with hexanes (28.4 L), CH_2Cl_2 (30 L), and EtOAc (24 L) to yield dark green hexanes (83.16 g), dark green CH_2Cl_2 (48.02 g), reddish-brown EtOAc (80.69 g), and reddish-brown aqueous (MeOH) (312.46 g) extracts. The stem CH_2Cl_2 extract (48.02 g) was fractionated by column chromatography (silica gel, hexanes−CH_2Cl_2, 70:30−30:70) to obtain 15 fractions. Fraction 2 (269.9 mg) was purified by column chromatography (CC, silica gel, hexanes−EtOAc, 99.7:0.3) to give three subfractions; the third subfraction (61 mg) was chromatographed (silica gel, hexanes−EtOAc, 99.7:0.3) to give 63.9 mg of a colorless solid of lupeol acetate. Fraction 4 (465.9 mg) was chromatographed (silica gel, hexanes−EtOAc, 98:2 to 95:5) to give three subfractions (4.1−4.3); subfraction 4.2 (242.2 mg) was recrystallized from hexanes−EtOAc (8:2) to give taraxerol (33.2 mg). Fraction 5 (938.6 mg) was recrystallized from hexanes−EtOAc (8:2) to give an additional quantity of taraxerol (67.7 mg), and the concentrated mother liquor was recrystallized from CH_2Cl_2−MeOH (4:6) to give lupeol (77.1 mg). Fraction 6 (939.5 mg) was purified by CC (silica gel, hexanes−EtOAc, 95:5 to 50:50) to give three subfractions (6.1−6.3); subfraction 6.2 (356.1 mg) after CC (silica gel, hexanes−CH_2Cl_2, 50:50, to CH_2Cl_2−MeOH, 99.9:0.5) gave a mixture of sitosterol and stigmasterol (142.8 mg). Fraction 8 (323.4 mg) was purified by CC (silica gel, hexanes−EtOAc, 80:20−50:50) to give three subfractions (8.1−8.3); subfraction 8.2 (44.2 mg) gave *p*-hydroxybenzaldehyde (9.3 mg). Subfraction 8.3 (14.0 mg) was separated by chromatography (RP-18, MeOH−H_2O, 70:30−100:0) to give scopoletin (5.9 mg). Fraction 10 (11.40 g) was fractionated (CC, silica gel, hexanes−EtOAc, 70:30, CH_2Cl_2−MeOH, 99.5:0.5) to give six subfractions (10.1−10.6); subfraction 10.2 (1.63 g) after CC (silica gel, CH_2Cl_2−MeOH, 99.7:0.3−96:4) gave five subfractions (10.2.1−10.2.5). Subfraction 10.2.2 (996.7 mg) after CC (RP-18, MeOH−H_2O, 65:35) and then silica gel (hexanes−EtOAc, 50:50) gave colorless compound **4** (58.8 mg); an aliquot of **4** after acetylation (using Ac_2O, pyridine) and purification (CC, silica gel, hexanes −EtOAc, 50:5) gave a colorless solid of **4a**. Subfraction 10.2.3 (76.7 mg) was chromatographed (silica gel, hexanes−EtOAc, 50:50) to give four subfractions; the third subfraction was purified by CC (silica gel, hexanes−EtOAc, 50:50) to give four subfractions, the second subfraction of which (31.5 mg) was purified by HPLC, RP-18, MeOH−H_2O, 60:40 to 100:0, TSP 2000, flow rate 1 mL/min, detected at 220 nm, to give **9**, t_R = 12.8 min (3.4 mg), and **8**, t_R = 13.7 min (11.2 mg). This subfraction containing a mixture of **8** and **9** (62.3 mg) was acetylated using Ac_2O−pyridine, the crude product (53.6

DOI: 10.1021/acs.jnatprod.6b00730
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mg) was fractionated using Sephadex LH-20 and MeOH, and the major fraction was further purified using HPLC, MeOH−H_2O, 60:40 to 100:0, to give **9a**, t_R = 19.7 min (4.7 mg), and **8a**, t_R = 20.8 min (8.4 mg). Subfraction 10.2.3.4 (79.3 mg) was fractionated (Sephadex LH-20, MeOH, and then RP-18, MeOH−H_2O, 50:50) to give lariciresinol (9.0 mg). Fraction 10.2.4 (202.8 mg) was purified by CC (Sephadex LH-20, MeOH) to obtain three subfractions (10.2.4.1−10.2.4.3); subfraction 10.2.4.2 (45.6 mg), after CC (RP-18, MeOH−H_2O, 60:40 to 70:30), gave a colorless solid (18.8 mg) of **3**. Subfraction 10.3 (1.01 g) was recrystallized (hexanes−CH_2Cl_2, 6:4) to give colorless needles (114.6 mg) of **1**. An aliquot of **1** (30 mg) was acetylated using Ac_2O−pyridine to obtain **1a** after purification (silica gel, hexanes−EtOAc, 50:50) (28.9 mg). Subfraction 10.4 (2.24 g) was chromatographed (silica gel, CH_2Cl_2−MeOH, 99.5:0.5−93:7) to give six subfractions (10.4.1−10.4.6). Subfraction 10.4.2 (282.5 mg) was recrystallized from hexanes−CH_2Cl_2 6:4 to give fine needles (47.1 mg) of **1**. Subfraction 10.4.4 (324.0 mg) was purified by CC (RP-18, MeOH−H_2O, 50:50) and then silica gel (hexanes−EtOAc, 45:65) to give five subfractions (10.4.4.1−10.4.4.5); subfraction 10.4.4.2 gave an additional quantity of **1** (20.7 mg), and subfraction 10.4.4.4 gave **2** (17.4 mg). Subfraction 10.5 (1.495 g) was subjected to CC (silica gel, hexanes−EtOAc, 20:80, to CH_2Cl_2−MeOH, 98:2) to give four subfractions (10.5.1−10.5.4). Subfraction 10.5.2 (1.17 g) was separated by chromatography (RP-18, MeOH−H_2O, 50:50−100:0) to obtain two subfractions (10.5.2.1−10.5.2.2); subfraction 10.5.2.1 (365.9 mg) was fractionated (CC, Sephadex LH-20, MeOH) to give three subfractions. Subfraction 10.5.2.1.2 (193.8 mg) after CC (RP-18, MeOH−H_2O, 60:40−100:0, and then Sephadex LH-20, MeOH) gave an additional quantity of a colorless solid (36.2 mg) of **2**. The fraction containing **2** as a major compound was acetylated using Ac_2O−pyridine to obtain **2a** as a colorless solid after CC (silica gel, hexanes−EtOAc, 50:50). Fraction 14 (11.35 g) was fractionated (CC, Sephadex LH-20, MeOH) to obtain three subfractions (14.1−14.3); subfraction 14.2 (8.0 g) was column chromatographed (silica gel, CH_2Cl_2−MeOH, 93:7−80:20) to give four subfractions (14.2.1−14.2.4). Subfraction 14.2.2 (1.73 g) after further CC [(RP-18 MeOH−H_2O, 60:40 to 100:0, then Sephadex LH-20 (MeOH)] and finally CC [(silica gel, CH_2Cl_2−MeOH, 93:7)] gave compound **7** (12.3 mg) and compound **5** (9.4 mg). Subfraction 14.2.3 (3.42 g) was subjected to CC (silica gel, CH_2Cl_2−MeOH, 95:5−90:10) to give four subfractions (14.2.3.1−14.2.3.4); subfraction 14.2.3.2 (351.2 mg) after CC (RP-18, MeOH−H_2O, 50:50, then Sephadex LH-20, MeOH) gave an additional quantity of **5** (29.5 mg). Subfraction 14.2.3.4 (763.4 mg) was subjected to CC (RP-18, MeOH−H_2O, 50:50) to obtain compound **6** (21.1 mg).

Acid Hydrolysis of 1. A mixture of compound **1** (6.5 mg) and 0.05 M HCl (1.5 mL) was refluxed at 60 °C for 4 h. After cooling, the reaction mixture was extracted with EtOAc (3 × 10 mL). The combined EtOAc extract was washed with water and concentrated to give the EtOAc extract (5.1 mg). Reversed-phase column chromatography (MeOH−H_2O, 40:60) of the extract gave the aglycone 12α,20α-epoxy-14β-hydroxypregn-5-en-11-one (3.4 mg, R_f 0.35) and D-diginose (1.8 mg, R_f 0.52) [silica gel TLC, thickness 0.2 mm, CH_2Cl_2−MeOH (94:6)]. The aqueous extract after neutralization and concentration gave an additional quantity of D-diginose (0.9 mg). The optical rotation value was measured after 24 h of dissolution in H_2O: D-diginose $[\alpha]^{26}_D$ +68 (*c* 0.1, H_2O) (lit.[13,14a] +59.6 (*c* 1.48, H_2O)).

Acid Hydrolysis of 5. A mixture of compound **5** (13.8 mg) and 0.05 M HCl (3.0 mL) was refluxed at 60 °C for 6 h. After cooling, the reaction mixture was extracted with EtOAc (3 × 10 mL). The combined EtOAc extract after usual workup (5.1 mg) and column chromatography (silica gel, CH_2Cl_2−MeOH, 98:2) gave 12α,20α-epoxy-14β-hydroxypregn-5-en-11-one (4.1 mg), R_f 0.35 (CH_2Cl_2−MeOH, 96:4): $[\alpha]^{26}_D$ −200 (*c* 0.4, $CHCl_3$); FT-IR (ATR) ν_{max} 3442, 2928, 2876, 1712, 1458, 1378, 1270, 1144, 1113, 1067, 1047, 1033, 1020, 973 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H 5.47 (d, *J* = 5.5 Hz, H-6), 3.90 (s, H-12), 3.53 (H-3), 3.46 (H-20), 2.36 (dt, *J* = 11.9, and 4.4 Hz, H-8); 2.35 (H-4), 2.27 (H-7), 2.14 (brt, *J* = 11.0 Hz, H-4), 2.02 (H-1), 2.02 (d, *J* = 12.3 Hz, H-9), 1.95 (H-16), 1.86 (H-2), 1.83 (H-17), 1.75 (H-7), 1.58 (H-15), 1.45(H-2), 1.39 s (H-19), 1.35 (H-1), 1.317 (d, *J* = 6.0 Hz, H-21), 1.27 (H-16), 1.03 s (H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 211.2 (C, C-11), 140.8 (C, C-5), 121.7 (CH, C-6), 91.5 (CH, C-12), 82.3 (CH, C-20), 82.2 (C, C-14), 71.6 (CH, C-3), 63.2 (C, C-13), 59.6 (CH, C-9), 58.4 (CH, C-17), 41.4 (CH_2, C-4), 39.2 (C, C-10), 37.3 (CH_2, C-1), 36.5 (CH, C-8), 32.4 (CH_2, C-15), 31.2 (CH_2, C-2), 28.1 (CH_2, C-7), 22.7 (CH_3, C-18), 20.2 (CH_3, C-21), 20.0 (CH_3, C-19); HRESIMS *m/z* 369.2026 $[M + Na]^+$ (calcd for $C_{21}H_{30}O_4Na$, 369.2034). The aqueous phase was neutralized with saturated $NaHCO_3$ and evaporated to give an aqueous extract (8.1 mg). Column chromatography (silica gel, CH_2Cl_2−MeOH, 92:8 to 75:25) of the crude extract gave D-diginose (0.9 mg, R_f 0.57), β-D-glucopyranosyl-(1→4)-D-diginopyranose (1.1 mg, R_f 0.15), and D-glucose (5.4 mg, R_f 0.03) [silica gel TLC, thickness 0.2 mm, CH_2Cl_2−MeOH (92:8)]. The optical rotation values were measured after 24 h of dissolution in H_2O: D-diginose $[\alpha]^{26}_D$ +59 (*c* 0.1, H_2O) (lit.[13,14a] +59.6 (*c* 1.48, H_2O); β-D-glucopyranosyl-(1→4)-D-diginopyranose, $[\alpha]^{26}_D$ +62 (*c* 0.1, H_2O) (lit.[15] +50.8 (*c* 1.3, MeOH); and D-glucose, $[\alpha]^{26}_D$ +59 (*c* 0.1, H_2O) (lit.[14a] +53.2 (*c* 0.1, H_2O).

3-β-O-12α,20α-Epoxy-14β-hydroxypregn-5-en-11-one β-D-diginopyranoside (1): colorless solid, mp 192−194 °C; $[\alpha]_D$ −182 (*c* 0.5, $CHCl_3$); FT-IR (ATR) ν_{max} 3463, 2936, 2890, 1711, 1661, 1445, 1367, 1322, 1269, 1194, 1167, 1129, 1102, 1060, 1031, 974, 893, 813, 784, 725, 658, 580 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; HRESIMS *m/z* 513.2828 $[M + Na]^+$ (calcd for $C_{28}H_{42}O_7Na$, 513.2816).

4′-O-Acetyl-3-β-O-12α,20α-epoxy-14β-hydroxypregn-5-en-11-one β-D-diginopyranoside (1a): $[\alpha]_D$ −132 (*c* 0.4, $CHCl_3$); FT-IR (ATR) ν_{max} 3445, 2967, 2956, 2930, 2889, 2841, 1735, 1711, 1439, 1379, 1374, 1360, 1235, 1172, 1140, 1112, 1059, 1034, 1019, 973, 951, 884, 863, 847, 814, 661 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table S1; HRESIMS *m/z* 555.2956 $[M + Na]^+$ (calcd for $C_{30}H_{44}O_8Na$, 555.2928).

12α,20α-Epoxy-8α,14β-dihydroxypregn-5-en-11-one 3-O-β-D-diginopyranoside (2): colorless solid, mp 146−148 °C; $[\alpha]_D$ −57 (*c* 0.7, $CHCl_3$); FT-IR (ATR) ν_{max} 3436, 2925, 2856, 1736, 1710, 1460, 1445, 1371, 1260, 1191, 1371, 1260, 1191, 1166, 1098, 1059, 1047, 1024, 975, 886, 864, 809, 727 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; HRESIMS *m/z* 529.2759 $[M + Na]^+$ (calcd for $C_{28}H_{42}O_8Na$ 529.2766).

4′-O-Acetyl-12α,20α-epoxy-8α,14β-dihydroxypregn-5-en-11-one 3-O-β-D-diginopyranoside (2a): colorless solid, mp 204−206 °C; $[\alpha]_D$ −84 (*c* 0.6, $CHCl_3$): FT-IR (ATR) ν_{max} 3518, 3426, 2965, 2928, 2872, 1747, 1698, 1457, 1439, 1378, 1367, 1342, 1169, 1227, 1132, 1103, 1063, 1022, 967, 979, 946, 915, 888, 860, 809, 728 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table S1; HRESIMS *m/z* 571.2898 $[M + Na]^+$ (calcd for $C_{30}H_{44}O_9Na$, 571.2877).

12α,20α-Epoxy-12β,14β-dihydroxypregn-5-en-11-one 3-O-β-D-diginopyranoside (3): $[\alpha]_D$ −49 (*c* 0.5, $CHCl_3$); FT-IR (ATR) ν_{max} 3468, 2965, 2933, 2867, 1717, 1458, 1442, 1375, 1268, 1189, 1166, 1118, 1098, 1062, 1030, 1017, 972, 941, 913, 886, 843, 816, 723 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; HRESIMS *m/z* 529.2779 $[M + Na]^+$ (calcd for $C_{28}H_{42}O_8Na$, 529.2766).

Kerriipregnane A 3-O-β-D-diginopyranoside (4): colorless solid, mp 158−162 °C; $[\alpha]_D$ −78 (*c* 0.5, $CHCl_3$); FT-IR (ATR) ν_{max} 3511, 2969, 2937, 2874, 1756, 1447, 1376, 1367, 1192, 1167, 1098, 1060, 1028, 979, 966, 937, 894, 851, 812, 719 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; HRESIMS *m/z* 527.2625 $[M + Na]^+$ (calcd for $C_{28}H_{40}O_8Na$, 527.2615).

4′-O-Acetylkerriipregnane A 3-O-β-D-diginopyranoside (4a): colorless solid, mp 172−173 °C; $[\alpha]_D$ −25 (*c* 0.8, $CHCl_3$); FT-IR (ATR) ν_{max} 2978, 2946, 2883, 1757, 1715, 1456, 1439, 1380, 1361, 1240, 1253, 1192, 1168, 1103, 1062, 1029, 961, 950, 918, 726 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table S1; HRESIMS *m/z* 569.2725 $[M + Na]^+$ (calcd for $C_{30}H_{42}O_9Na$, 569.2715).

12α,20α-Epoxy-14β-hydroxypregn-5-en-11-one 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside (5): $[\alpha]_D$ −79 (*c* 0.7, $CHCl_3$); FT-IR (ATR) ν_{max} 3373, 2926, 2870, 2855, 1741, 1711,

DOI: 10.1021/acs.jnatprod.6b00730
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1655, 1596, 1451, 1369, 1322, 1271, 1220, 1195, 1166, 1099, 1057, 1026, 1017, 972, 889, 863, 811 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table 2; HRESIMS m/z 675.3356 $[M + Na]^+$ (calcd for $C_{34}H_{52}O_{12}Na$, 675.3342).

*12α,20α-Epoxy-8α,14β-dihydroxypregn-5-en-11-one 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside (**6**).* colorless solid, mp 190−194 °C; $[\alpha]_D$ −90 (c 0.5, $CHCl_3$); FT-IR (ATR) ν_{max}3406, 2973, 2935, 2871, 1700, 1647, 1596, 1456, 1445, 1370, 1320, 1276, 1223, 1199, 1167, 1153, 1100, 1071, 1051, 1022, 994, 977, 954, 944, 916, 892, 863 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table 2; HRESIMS m/z 691.3310 $[M + Na]^+$ (calcd for $C_{34}H_{52}O_{13}Na$ 691.3300).

*Kerriipregnane A 3-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside (**7**):* colorless solid, mp 158−162 °C; FT-IR (ATR) ν_{max} 3384, 2970, 2934, 2872,1761, 1732, 1456, 1442, 1369, 1170, 1099, 1056, 1020 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table 2; HRESIMS m/z 689.3204 $[M + Na]^+$ (calcd for $C_{34}H_{50}O_{13}Na$, 689.3134).

*Kerriipregnane B 3-O-β-D-diginopyranoside (**8**):* colorless solid, mp 132−134 °C; $[\alpha]_D$ −48 (c 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data see Table 3; HRESIMS m/z 559.2881 $[M + Na]^+$ (calcd for $C_{29}H_{44}O_9Na$, 559.2871).

*4′-O-Acetylkerriipregnane B 3-O-β-D-diginopyranoside (**8a**):* ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 3; HRESIMS m/z 601.3006 $[M + Na]^+$ (calcd for $C_{31}H_{46}O_{10}Na$, 601.2983).

*13-epi-Kerriipregnane B 3-O-β-D-diginopyranoside (**9**):* $[\alpha]_D$ −46.46 (c 0.24, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 3; HRESIMS m/z [M + Na] 559.2888 (calcd for $C_{29}H_{44}O_9Na$ 559.2871).

*4′-O-Acetyl-13-epi-kerriipregnane B 3-O-β-D-diginopyranoside (**9a**):* FT-IR (ATR) ν_{max} 3445, 2966, 2929, 2891, 2842, 1734, 1711, 1438, 1380, 1235, 1170, 1138, 1111, 1059, 1034, 1019, 973, 862 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 3; HRESIMS m/z 601.3023 $[M + Na]^+$ (calcd for $C_{31}H_{46}O_{10}Na$, 601.2983).

X-ray Crystallographic Analysis of Compounds 1, 4a, and 8a. X-ray analysis was carried out on a Bruker-Nonius kappaCCD diffractometer using a graphite monochromator with Mo Kα radiation (λ = 0.710 73 Å) at 298(2) K. The structures were solved by direct methods using SIR97[17] and refined with full-matrix least-squares calculation on F^2 using SHELXL-97.[18] The crystallographic data have been deposited at the Cambridge Crystallographic Data Centre under the reference numbers CCDC 1484810−1484812.

*Crystal data for **1**:* $2(C_{28}H_{42}O_7)$ $3(H_2O)$, MW = 1035.3, 0.15 × 0.15 × 0.20 mm^3, monoclinic, space group $P2_1$ (No. 4), a = 7.2520(1) Å, b = 35.8010(5) Å, c = 10.7280(2) Å, β = 90.0590(5)°, V = 2785.30(8) $Å^3$, Z = 2, T = 298(2) K, μ(Mo Kα) = 0.090 mm^{-1}, D_x = 1.235 g/cm^3, reflections measured/unique: 16 175/3306, observed [$I > 2\sigma(I)$]: 3154, R_1 = 0.0464, wR_2 = 0.1250 (all data).

*Crystal data for **4a**:* $C_{30}H_{42}O_9$, MW = 546.64, 0.25 × 0.25 × 0.30 mm^3, monoclinic, space group $P2_1$ (No. 4) a = 12.0520(6), Å, b = 8.5300(4) Å, c = 14.5650(7) Å, β = 95.408(3)°, V = 1490.67(12) $Å^3$, Z = 2, T = 298(2) K, μ(Mo Kα) = 0.090 mm^{-1}, D_x = 1.218 g/cm^3, reflections measured/unique: 6495/1868, observed [$I > 2\sigma(I)$]: 1439, R_1 = 0.0577, wR_2 = 0.1710 (all data).

*Crystal data for **8a**:* $2(C_{30}H_{46}O_{710})$, MW = 578.68, 0.15 × 0.15 × 0.30 mm^3, orthorhombic, space group $P2_12_12$ (No. 18), a = 27.6220(4) Å, b = 19.96800(10) Å, c = 5.6990(5) Å, V = 3143.3(3) $Å^3$, Z = 4, T = 298(2) K, μ(Mo Kα) = 0.090 mm^{-1}, D_x = 1.223 g/cm^3, reflections measured/unique: 5050/4650, observed [$I > 2\sigma(I)$]: 4200, R_1 = 0.0520, wR_2 = 0.1346 (all data).

Bioassays. The assay for anti-inflammatory activity based on the inhibition of NO production in RAW264.7 cells was conducted using a previously published protocol.[15]

To acquire the mechanism of action of the cytokine release of **9a**, assays for the mRNA expression of iNOS and COX-2 were performed. The total RNA was isolated from RAW264.7 cells and was harvested after 20 h of incubation with samples at various concentrations (3, 10, 30, 100 μM) using the RNeasy mini kit (Qiagen Operon Co. Ltd., USA). The total RNA from each sample was used for cDNA synthesis using a first-strand cDNA synthesis kit (Rever Tra Ace-α, TOYOBO Co., Ltd., Japan), followed by RT-PCR (Rever Tra Dash, TOYOBO Co., Ltd., Japan). The primers for iNOS and COX-2 were used (forward primer for iNOS: 5′-ATCTGGATCAGGAACCTGAA-3′ and its reverse primer: 5′-CCTTTTTTGCCCCATAGGAA-3′; forward primer for COX-2:5′-GGAGAGACTATCAAGATA-GTGATC-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTT-TACAGCTC-3′; forward primer for β-actin (an internal standard): 5′-TGTGATGGTGGGAATGGGTCAG-3′ and reverse primer: 5′-TTTGATGTCACGCACGATTTCC-3′). The solution for cDNA synthesis consisted of 11 μL of RNA solution, 4 μL of 5× RT buffer, 2 μL of dNTP mixture (10 mM), 1 μL of RNase inhibitor (10 U/μL), 1 μL of Oligo(dT)20, and 1 μL of Rever Tra Ace (reverese transcriptase enzyme) for a 20 μL reaction. The conditions for cDNA synthesis were as follows: 42 °C for 20 min, 99 °C for 5 min, and 4 °C for 5 min. After that, 1/10 the volume (2 μL) of the cDNA product was further used for PCR. The PCR mixture consisted of 2 μL of RT reaction mixture (cDNA product): 85 μL of sterilized water, 10 μL of 10× PCR buffer, 1 μL of forward primer (10 pmol/ μL), 1 μL of reverse primer (10 pmol/ μL), and 1 μL of KOD Dash (polymerase enzyme) for a final volume of 100 μL. The conditions for PCR were as follows: denaturation at 94 °C for 1 min, 98 °C for 30 s, 55 °C for 30 s, and 74 °C for 1 min (30 cycles). The PCR products were analyzed in 1.2% agarose gel electrophoresis and visualized by SYBR safe staining and UV irradiation under a wavelength of 312 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00730.

HMBC correlations of compounds **1**−**4** and **8** (PDF)
X-ray data of **1** (CIF)
X-ray data of **4a** (CIF)
X-ray data of **8a** (CIF)
^1H and ^{13}C NMR spectroscopic data of **1a**, **2a**, and **4a** in $CDCl_3$ (PDF)
^1H and ^{13}C NMR spectra of compounds **1**−**9**; 2D NMR spectra of **1**, **4**, and **8** (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to the Thailand Research Fund (BRG5580012) and Ramkhamhaeng University for financial support. C.S. acknowledges the Royal Golden Jubilee Ph.D. Program, Thailand Research Fund, and the Center of Excellence for Innovation in Chemistry (PERCH−CIC), the Commission on Higher Education, the Ministry of Education, for a scholarship. P.K. gratefully acknowledges the support from Mahidol University and the Office of the Higher Education Commission under the National Research Universities Initiative. We acknowledge Mr. M. Taegyong, Mr. S. Singha, Miss S. Kruakaew, and the late Mr. W. Komkhunthot for technical assistance.

DOI: 10.1021/acs.jnatprod.6b00730
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DOI: 10.1021/acs.jnatprod.6b00730
J. Nat. Prod. XXXX, XXX, XXX−XXX