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Norstaminane- and isopimarane-type diterpenes of *Orthosiphon stamineus* from Okinawa

Suresh Awale,[a] Yasuhiro Tezuka,[a] Arjun H. Banskota,[a] Seikichi Shimoji,[b] Kazuhiko Taira[c] and Shigetoshi Kadota[a,*]

[a]*Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630-Sugitani, Toyama 930-0194, Japan*
[b]*Okinawa Health Food Development Cooperative, 1075 Tsuhako aza Sashiki, Okinawa 901-1414, Japan*
[c]*Faculty of Education, University of the Ryukyus, 1-Senbaru, Nishihara-cho, Okinawa 903-0129, Japan*

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Abstract—Nine novel highly oxygenated and structurally diverse diterpenes, named norstaminolactone A (**1**), norstaminols B and C (**2** and **3**), secoorthosiphols A–C (**4**–**6**) and orthosiphols R–T (**7**–**9**) have been isolated from the aerial part of *Orthosiphon stamineus* cultivated in Okinawa Prefecture, Japan. Norstaminolactone A (**1**) is the first representative of a biogenetically unusual norstaminane-type diterpene bearing a nitrogen atom. Norstaminol C (**3**) possessed a framework presumed to be biosynthesized from the staminane-type diterpene. Secoorthosiphols A–C (**4**–**6**) possessed an unprecedented structural feature of the opened ring A system, encountered for the first time in isopimarane-type diterpenes. Secoorothosiphol C (**6**) also represents the first example of biogenetically unique and unconventional secoisopimarane-type diterpene bearing a cyano group. Norstaminolactone A (**1**) showed a potent antiproliferative activity with an IC_{50} value of 2.16 μg/mL against highly liver metastatic colon 26-L5 carcinoma cell line.

1. Introduction

Traditional medicines are widely used alongside with modern medicine in many countries of Southeast Asia and play an important role in promoting a health care system.[1] *Orthosiphon* (*O.*) *stamineus* Benth. [syn.: *O. aristatus* (Bl.) Miq., *O. grandiflorus* Bold., *O. spicatus* (Thumb) Bak.; Lamiaceae] is one of the popular traditional folk medicine extensively used in Southeast Asia for the treatment of wide range of diseases: in Indonesia for rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhea, syphilus, renal calculus, gallstone etc.;[2] in Vietnam for urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice and biliary lithiasis;[3] and in Myanmar to alleviate diabetes, urinary tract and renal diseases.[4] Owing to its beneficial pharmaceutical utility, it is under systematic cultivation in Okinawa Prefecture, Japan and is locally known as 'Neko no hige' or, 'Kumis kucing' both meaning 'Cats whisker', and consumed as a healthy Java tea to facilitate body detoxification. In our search for cancer antiproliferative agents from natural sources, we isolated a series of highly oxygenated isopimarane-type diterpenes and the diterpenes with a novel carbon-framework named 'staminane' from *Orthosiphon stamineus* of Vietnam and Myanmar.[5–8] Likewise, from Indonesian *O. stamineus*, neoorthosiphols A and B with the 'staminane' carbon-framework were reported by Shibuya et al.[9] However, there are no reports on the staminane-type diterpenes in *O. stamineus* from Okinawa, though they have been reported to contain isopimarane-type diterpenes, orthosiphols A, B, D and E.[10,11] Thus, we have investigated the constituents of *O. stamineus* cultivated in Okinawa Prefecture and isolated nine novel highly oxygenated diterpenes having biogenetically diverse structures based on the staminane and isopimarane carbon-frameworks. We herein report the isolation and structure elucidation of the new diterpenes by spectroscopic techniques as well as chemical methods, together with their antiproliferative activities (Chart 1).

2. Results and discussion

Air-dried aerial parts of *O. stamineus* from Okinawa were extracted with refluxing MeOH and the MeOH extract was successively partitioned into hexane, $CHCl_3$, EtOAc, BuOH and H_2O fractions. The $CHCl_3$ fraction was subjected to a series of chromatographic separation and preparative TLC to afford nine new highly oxygenated diterpenes together with nine known diterpenes. The new diterpenes were one 16-norstaminane-type diterpene named norstaminolactone A (**1**), two 14-norstaminane-type diterpenes named norstaminolactone A (**1**) and norstaminols B (**2**) and C (**3**), three secoisopimarane-type dieterpenes named secoorthosiphols A–C (**4**–**6**) and three isopimarane-type

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* Corresponding author. Tel.: +81-76-434-2281x2825; fax: +81-76-434-5059; e-mail: kadota@ms.toyama-mpu.ac.jp

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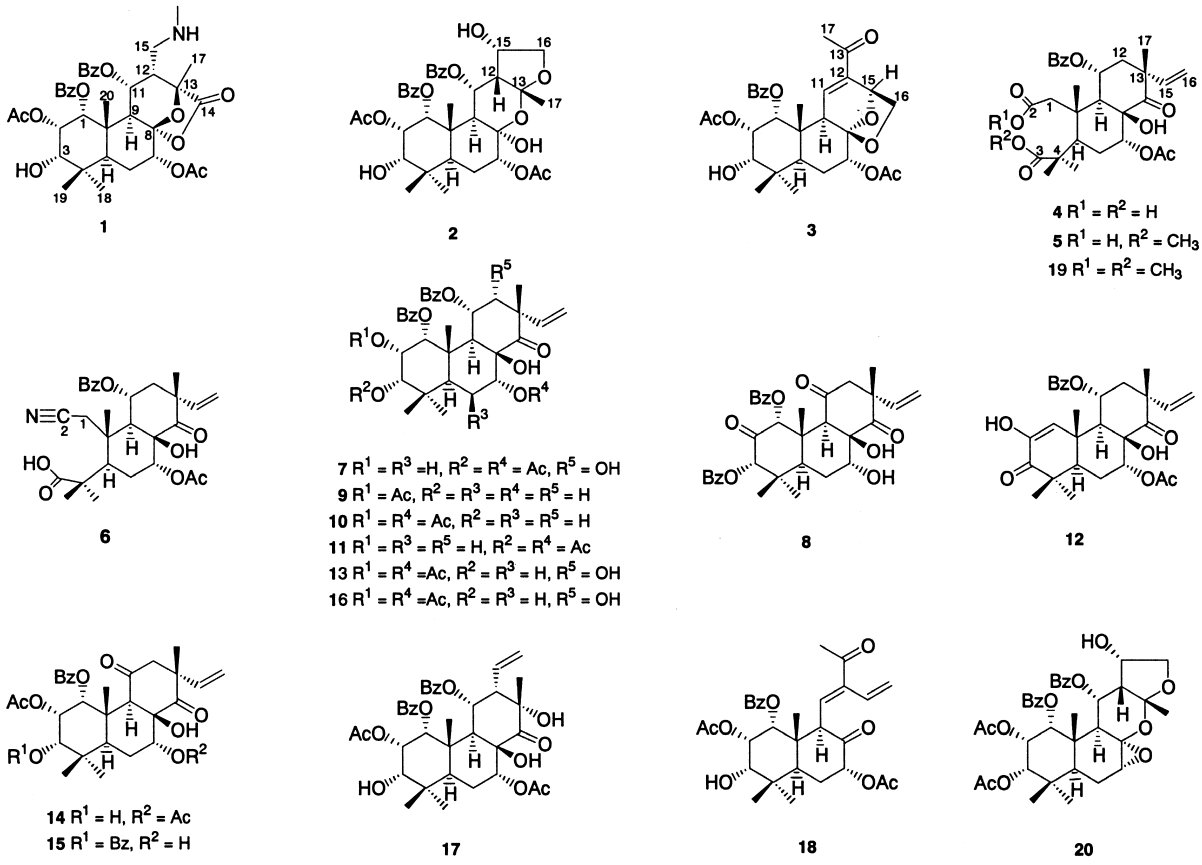


Chart 1. Structures of the compounds isolated from *O. stamineus* cultivated in Okinawa Prefecture, Japan.

diterpenes named orthospihols R–T (**7**–**9**), while the known compounds were identified, by analysis of their spectroscopic data and comparison with literature data, to be orthosiphols A[10] (**10**), B[10] (**11**), E[11] (**12**), L–N[7] (**13**–**15**) and P[8] (**16**), neoorthosiphol A[9] (**17**) and norstaminone A[7] (**18**).

Norstaminolactone A (**1**) was obtained as a colorless amorphous solid. It showed a positive reaction with the Dragendorff reagent, suggesting that it contains a nitrogen atom in the molecule. The negative ion HRFABMS showed a quasimolecular ion at *m/z* 706.2831 $(M-H)^-$, consistent with the molecular formula $C_{38}H_{44}O_{12}N$. The IR spectrum of **1** showed absorptions of amino (3250, 1610 cm^{-1}), hydroxyl (3550 cm^{-1}), ester carbonyl (1740 cm^{-1}), γ-lactone carbonyl (1800 cm^{-1}) and phenyl (1600, 1450 cm^{-1}) groups. The ^1H NMR spectrum of **1** displayed signals due to four tertiary methyls and five oxygen-substituted and two aliphatic methines, together with those of two acetyl and two benzoyl groups (Table 1), while its ^{13}C NMR spectrum revealed the signals of four ester carbonyls, a lactone carbonyl, a ketal and six oxygen-substituted carbons, one methylamino carbon and two oxygen-non-substituted quaternary carbons (Table 1). Excluding the ^{13}C NMR signals for two benzoyl, two acetyl and one methylamino group, **1** possessed only 19 carbon signals in its main carbon framework, suggesting it to be a norditerpene.

The partial structures obtained by the analysis of the COSY and HMQC spectra were connected based on the long-range correlations observed in the HMBC spectrum (Fig. 1a). Significant long-range correlations of the tertiary methyl protons H_3-18, H_3-19 and H_3-20 in the HMBC spectrum confirmed the structure of rings A and B, while rings C and D were constructed based on the HMBC correlations of H-9, H-11 and H_3-17. The locations of the ketal and lactone carbonyl carbons were established to be at C-8 and at C-14, respectively, from the correlations between the ketal carbon (δ_C 107.0) and H-9 and H-11 and between the lactone carbonyl carbon (δ_C 172.6) and H-12 and H_3-17, respectively. The presence of a methylamino group at C-15 was determined based on the ^1H and ^{13}C NMR chemical shifts for C-15 and N–CH_3 (C-15: δ_H 2.73, δ_C 45.6; N–CH_3: δ_H 2.40, δ_C 47.9). On the other hand, the locations of the two benzoyl and two acetyl groups were determined to be at C-1 and C-11 and at C-2 and C-7, respectively, based on the HMBC correlations between the ester carbonyl carbon at δ_C 164.4 (1-OCO) and the protons at δ_H 5.60 (H-1) and 7.77 (1-OCOPh), between the ester carbonyl carbon at δ_C 170.1 (2-OCO) and the protons at δ_H 5.49 (H-2) and 2.09 (2-$OCOCH_3$), between the ester carbonyl carbon at δ_C 169.0 (7-OCO) and the protons at δ_H 5.27 (H-7) and 2.21 (7-$OCOCH_3$) and between the ester carbonyl carbon at δ_C 163.8 (11-OCO) and the protons at δ_H 5.58 (H-11) and 7.60 (11-OCOPh).

The relative stereochemistry of **1** was assigned on the basis of the ROESY correlations and the coupling constant data. The ROESY correlations H-2/H-3, H-2/H_3-19, H-2/H_3-20, H_3-19/H_3-20 and H-5/H-9 indicated rings A and B to have a chair conformation (Fig. 1b). On the other hand, the ROESY

Table 1. ^{1}H and ^{13}C NMR data for compounds **1**–**3** in $CDCl_3$ (*J* values in parentheses)

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.60 d (2.5)	73.7	5.37 d (3)	73.0	5.55 d (2.8)	74.1
2	5.49 t (2.5)	67.5	5.45 t (3)	67.5	5.46 t (2.8)	67.8
3	3.60 br s	77.2	3.56 d (3)	77.2	3.65 br s	77.2
4		38.0		38.1		38.3
5	2.52 d (12.7)	34.4	2.47 dd (10.5, 4.1)	35.1	2.48 dd (12.2, 4.3)	34.6
6	2.06 m; 1.90 m	23.1	1.90 m	23.6	1.90 m	24.4
7	5.27 br s	71.2	5.04 t (2.6)	71.2	5.10 t (2.6)	71.9
8		107.0		93.9		104.6
9	2.57 s	44.9	2.66 d (3.8)	46.3	2.56 d (4.2)	45.7
10		43.5		41.9		43.4
11	5.58 d (4.6)	65.5	5.58 t (3.8)	64.7	6.66 d (4.2)	137.2
12	1.74 d (4.6)	50.1	2.37 t (3.8)	43.6		141.6
13		79.9		104.7		196.4
14		172.6				
15	2.73 m	45.6	4.51 br s	72.6	5.32 d (3.9)	73.5
16			3.80 d (9.4)	72.8	3.69 dd (6.8, 3.9)	72.2
			3.70 dd (9.4, 1.9)		3.57 d (6.8)	
17	1.64 s	19.0	1.65 s	21.5	2.30 s	24.8
18	1.12 s	28.9	1.11 s	22.4	1.02 s	22.1
19	1.34 s	22.2	1.01 s	28.8	1.18 s	28.6
20	1.35 s	16.9	1.34 s	16.0	1.26 s	16.7
$N–CH_3$	2.40 m	47.9				
1-OBz						
1′		129.8		129.5		129.3
2′,6′	7.77 d (7.6)	129.3	7.59 d (7.5)	129.7	8.12 d (7.8)	129.9
3′,5′	7.21 t (7.6)	128.1	7.11 t (7.5)	128.5	7.52 t (7.8)	128.9
4′	7.40 t (7.6)	132.9	7.43 t (7.5)	133.0	7.65 t (7.8)	133.8
7′		164.4		164.4		165.7
11-OBz						
1″		129.7		129.3		
2″,6″	7.60 d (7.3)	129.6	7.60 d (7.6)	129.4		
3″,5″	7.09 t (7.3)	128.2	7.30 t (7.6)	128.1		
4″	7.26 t (7.3)	133.0	7.55 t (7.6)	132.7		
7″		163.8		165.6		
2-OAc						
$CO\mathit{CH}_3$	2.09	21.1	1.92 s	20.8	2.03 s	20.9
$\mathit{C}OCH_3$		170.1		169.8		169.9
7-OAc						
$CO\mathit{CH}_3$	2.21	21.0	2.23 s	21.4	2.11 s	21.1
$\mathit{C}OCH_3$		169.0		169.4		169.5

correlations H_3-20/H-11, H-11/H-12 and H-12/H-17 and the small coupling constant between H-9 and H-11 (J=4.6 Hz) indicated a boat conformation of ring C and a *gauche* conformation of H-11 and H-9. On the other hand, molecular model analysis of **1** by semiemperical MM2 calculation indicated that, when the lactone bridge is α-oriented, a ring C should have boat conformation having small dihedral angle between H-9 and H-11 (92.8°), while that for β-oriented lactone bridge should have chair conformation with almost anti relationship between H-9 and H-11 (dihedral angle, 178.5°). Since the coupling constant value between H-9 and H-11 is small, the lactone bridge in ring D must be α-oriented.

Norstaminol B (**2**) was obtained as a colorless amorphous solid and its negative ion HRFABMS showed the quasi-molecular ion at m/z 695.2701 $(M-H)^-$, corresponding to the molecular formula $C_{37}H_{44}O_{13}$. The IR spectrum of **2** showed absorptions of hydroxyl (3550 cm^{-1}), ester carbonyl (1705 cm^{-1}) and phenyl (1600, 1450 cm^{-1}) groups. The ^{1}H NMR spectrum of **2** displayed signals due to four tertiary methyls and six oxygen-substituted and three oxygen-unsubstituted methines, together with those of two acetyl and two benzoyl groups, while its ^{13}C NMR spectrum revealed the signals of two ketal, four ester carbonyl, seven monooxygenated sp^3 carbons and two unoxygenated sp^3 quaternary carbons (Table 1). Excluding the ^{13}C NMR signals for two benzoyl and two acetyl groups, **2** possessed only 19 carbon signals in its main carbon framework, suggesting it to be a norditerpene. These ^{1}H and ^{13}C NMR data were similar to those of norstaminol A[6] (**20**). However, analysis of the COSY and HMQC spectra indicated a downfield shift of H-3 (δ_H 3.56) and an upfield shift of H-7 (δ_H 5.04) in **2**, compared to those of **20** (H-3, δ_H 5.05; H-7, δ_H 3.80). Thus, the hydroxyl and acetyl groups were located at C-3 and C-7, respectively, and the epoxide ring in **20** should be opened in **2**, which was confirmed by the HMBC correlations (Fig. 2a). Significant ROESY correlations H-2/H_3-20, H_3-19/H_3-20 and H-5/H9 indicated rings A and B to have chair conformations (Fig. 2b), while the correlations H-11/H_3-20, H-11/H-12, H-11/H_3-17 and H-12/H-15 indicated rings C and D to be in boat and envelop conformations, respectively. These ROESY correlations, as well as the small coupling constants for H-9 (d, J=3.8 Hz), H-11 (t, J=3.8 Hz) and H-12 (t, J=3.8 Hz), indicated the hydroxyl group at C-8 to be α-oriented; consideration of the Drieding

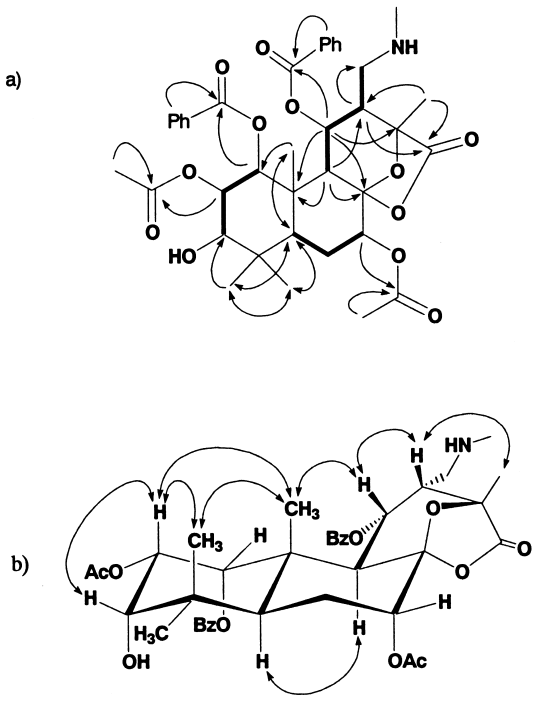


Figure 1. (a) Partial structures (bold line) deduced by the COSY and HMQC spectra and significant HMBC correlations (arrow) and (b) ROESY correlations for **1**.

stereomodel indicated that a β-oriented hydroxyl group at C-8 leads to almost anti relationship between H-9 and H-11 with a larger coupling constant. Thus, the structure of norstaminol B was assigned as **2**.

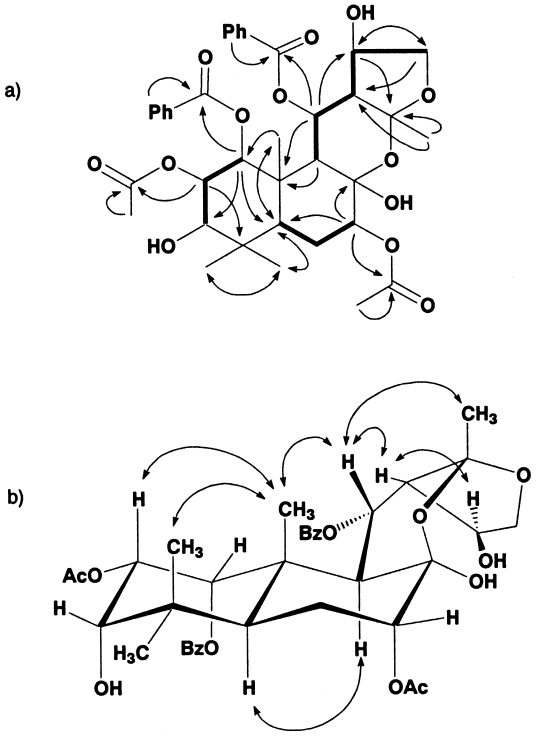


Figure 2. (a) Partial structures (bold line) deduced by the COSY and HMQC spectra and significant HMBC correlations (arrow) and (b) ROESY correlations for **2**.

Norstaminol C (**3**) was determined to have the molecular formula $C_{30}H_{36}O_{10}$ by HRFABMS. The IR spectrum of **3** showed the absorptions of hydroxyl (3500 cm^{-1}), α,β-unsaturated carbonyl (1710, 1675 cm^{-1}) and phenyl (1590, 1450 cm^{-1}) groups. The ^1H NMR spectrum of **3** displayed signals due to three tertiary methyls, five oxygen-substituted and two oxygen-nonsubstituted methines and a trisubstituted-olefinic proton, together with those of a benzoyl and two acetyl groups (Table 1). While, its ^{13}C NMR spectrum showed the signals of 19 carbons in its main carbon framework, including those of a ketone carbonyl, five monooxygenated sp^3 carbons, two olefinic carbons and a ketal carbon, excluding those for a benzoyl and two acetyl groups (Table 1). Thus, norstaminol C (**3**) also should be a norditerpene having a benzoyl and two acetyl groups.

The partial structures were obtained from the COSY and HMQC spectra and were connected by the HMBC correlations (Fig. 3a). Significant long-range correlations of the tertiary methyls H_3-18, H_3-19 and H_3-20 and those of the ester carbonyl carbons indicated rings A and B to have the same structure and substituents as in **1**. The structures of rings C and D were determined on the basis of the HMBC correlations for H-11, H-15, H-16 and H_3-17 (Fig. 3a). The relative stereochemistry of **3** was elucidated from the ROESY experiment (Fig. 3b) and the biogenetic considerations. The ROESY correlations H-2/H_3-19, H-2/H_3-20 and H-5/H-9 indicated that rings A and B have a chair conformation and that the ring junction is *trans*, while the orientation of H-15 was assumed to be α from a biogenetic consideration (Scheme 1) (vide infra). From these data, the structure of norstaminol C was concluded as **3**.

The molecular formula of secoorthosiphol A (**4**) was determined by a negative ion HRFABMS measurement to be $C_{29}H_{36}O_{10}$. The IR spectrum of **4** showed absorptions due to hydroxyl, ester carbonyl and phenyl groups. The ^1H NMR spectrum of **4** (Table 2) revealed signals due to four tertiary methyls, a vinyl, two oxygen-substituted methines and three aliphatic methylenes, together with those of an acetyl and a benzoyl group. The ^{13}C NMR spectrum (Table 2) indicated the presence of a ketone, two acid carbonyls, two ester carbonyls, two oxygen-substituted methines and an oxygen-substituted quaternary carbon. The partial structures were deduced by the analysis of the COSY and HMQC spectra and connected based on the long-range correlations observed in the HMBC spectrum (Fig. 4). Significant correlations were observed between the acid carbonyl carbon at δ_C 176.8 (C-2) and the protons at δ_H 2.63 and 2.17 (H_2-1) and between the acid carbonyl carbon at δ_C 185.6 (C-3) and the tertiary methyl protons H_3-18 and H_3-19, allowing the locations of two carboxylic acid groups. Similarly, correlations of the ester carbonyl carbon at δ_C 169.6 (7-OCO) with the protons at δ_H 2.07 (7-OCOCH_3) and 5.34 (H-7) and of the ester carbonyl carbon at δ_C 165.5 (11-OCO) with the protons at δ_H7.80 (H-2′,6′) and 5.53 (H-11) suggested the locations of the acetyl and benzoyl groups to be at C-7 and at C-11, respectively. These data leads to a planar structure of **4** (Fig. 4) having opened ring A system with dicarboxylic functionality.

The open and dicarboxylic nature of ring A was further

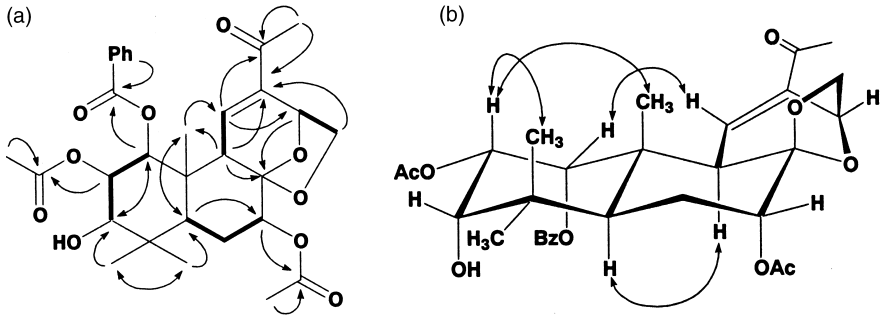


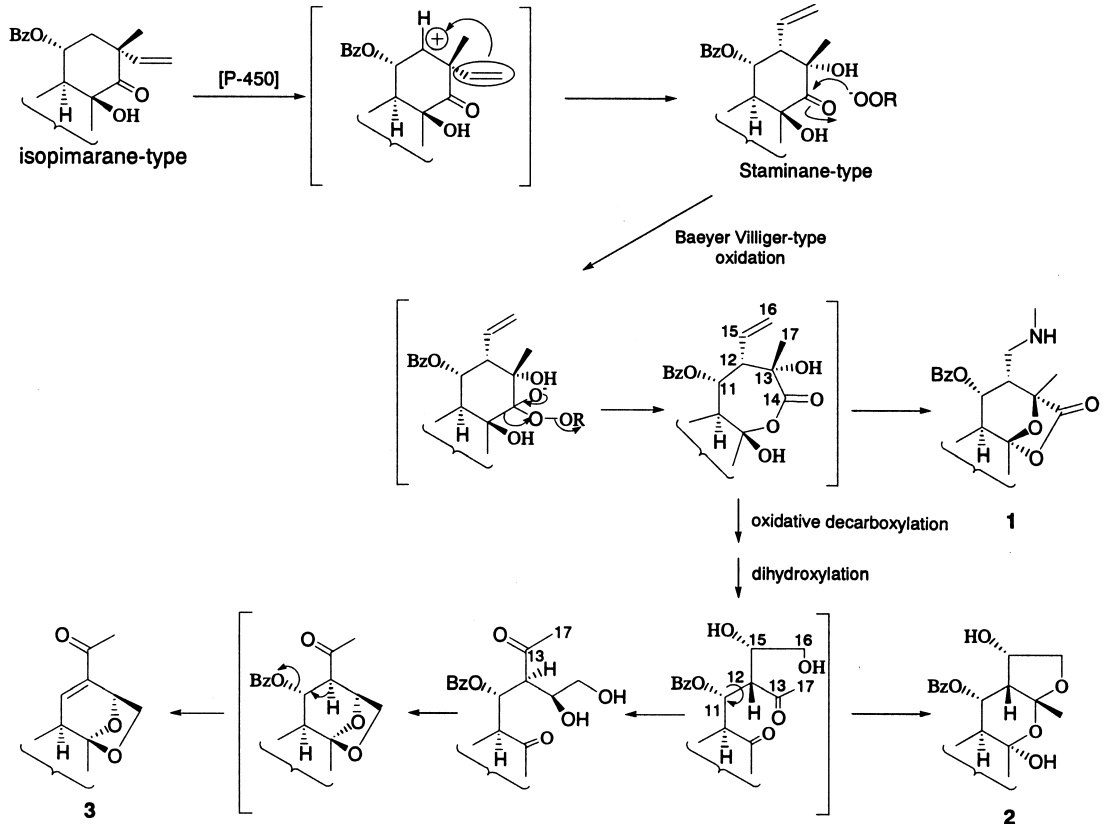
Figure 3. (a) Partial structures (bold line) deduced by the COSY and HMQC spectra and significant HMBC correlations (arrow) for **3**. (b) ROESY correlations for **3**.

confirmed by a methylation reaction; treatment of **4** with diazomethane gave a secoorthosiphol A dimethylester [**19**; *m/z* 571 $(M-H)^-$; δ_H 3.53, 2.96; δ_C 51.8, 50.0].

The relative stereochemistry of **4** was determined by the ROESY spectrum and the interpretation of coupling constant data. The large coupling constants for H-5 ($J_{5,6ax}$=12.7 Hz), H-9 and H-11 ($J_{9,11}$=7.1 Hz) indicated them to be axial, while the small ones for H-7 ($J_{6eq,7}$=2.4 Hz, $J_{6ax,7}$=0 Hz) indicated its equatorial nature. On the other hand, significant ROESY correlations observed between H_3-20 and H-11, between H-5 and H-9 and between H-11 and H_3-17 suggested rings B and C to have chair conformations. Thus, the structure of secoorthosiphol A was concluded to be **4**.

The ^1H and ^{13}C NMR spectra of secoorthosiphol B (**5**) were almost superimposable to those of **4**, except for the presence of one more methoxyl signal (δ_H 3.57, δ_C 52.0) (Table 2). This was consistent with the molecular formula $C_{30}H_{38}O_{10}$ determined by HRFABMS. The location of the methoxyl group was determined to be at C-3 based on the significant HMBC correlations of the ester carbonyl carbon at δ_C 179.1 (C-3) with the methoxyl protons at δ_H 3.57 and two tertiary methyl protons at δ_H 1.18 (H_3-18) and 1.20 (H_3-19). Thus, **5** was determined as 3-*O*-methylate of **4**.

Secoorthosiphol C (**6**) displayed a quasimolecular ion $(M-H)^-$ at *m/z* 524.2281, consistent with the molecular formula $C_{29}H_{36}NO_8$, indicating the presence of one nitrogen atom in **6**. The IR spectrum of **6** showed a characteristic sharp absorption for a cyano group (2250 cm^{-1}) together with the absorptions for hydroxyl, ester carbonyl and phenyl groups. The ^1H NMR spectrum of **6** was similar to that of **4**,



Scheme 1. Possible biogenetic pathways of novel staminane-type diterpenes.

Table 2. ^{1}H and ^{13}C NMR data for compounds **4**–**6** in $CDCl_3$ (J values in parentheses)

Position	**4**		**5**		**6**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.63 d (20.3)	39.7	2.32 d (20.3)	39.5	2.57 d (18.2)	29.3
	2.17 d (20.3)		2.22 d (20.3)		2.28 d (18.2)	
2		176.8		171.1		117.8
3		185.6		179.1		181.3
4		44.8		45.3		46.5
5	3.08 dd (12.7, 3.1)	40.6	3.05 dd (15.2, 2.7)	41.0	2.58 dd (15.2, 2.6)	42.7
6	2.08 m	22.8	2.07 m	23.4	2.16 td (15.2, 2.6)	24.5
	2.04 m		1.98 dt (15.2, 2.7)		1.97 dt (15.2, 2.6)	
7	5.34 br s	71.2	5.30 t (2.7)	71.0	5.31 br s	70.2
8		75.0		75.0		74.6
9	3.58 d (7.1)	42.5	3.78 d (7.3)	41.4	3.11 d (7.3)	44.3
10		44.2		44.2		44.2
11	5.53 t (7.1)	69.3	5.60 dd (7.3)	69.3	5.64 t (7.3)	68.7
12	2.54 dd (15.4, 7.1)	39.4	2.53 dd (15.6, 7.3)	39.0	2.58 dd (18.1, 7.3)	39.4
	2.06 d(15.4)		2.09 m		2.14 d (18.4)	
13		47.8		47.8		47.8
14		209.2		209.3		208.6
15	5.92 dd (17.6, 10.8)	140.7	5.95 dd (17.6, 10.8)	140.4	6.01dd (17.8, 10.7)	140.0
16	5.15 d (10.8)	114.4	5.15 d (10.8)	114.5	5.22 d (10.7)	115.2
	4.96 d (17.6)		4.98 d (17.6)		4.99 d (17.8)	
17	1.15 s	25.6	1.18 s	25.5	1.19 s	25.7
18[a]	1.20 s	28.8	1.18 s	27.9	1.29 s	25.3
19[a]	1.11 s	21.9	1.20 s	23.5	1.35 s	23.2
20	1.26 s	20.9	1.20 s	19.9	1.30 s	18.7
7-OAc						
$CO\mathit{CH}_3$	2.07 s	20.7	2.09 s	20.9	2.06 s	20.8
$\mathit{C}OCH_3$		169.6		169.3		169.4
11-OBz						
1′		130.3		131.2		129.4
2′,6′	7.80 d (8.1)	129.4	8.03 dd (7.6, 1.5)	129.5	8.20 d (7.5)	130.2
3′,5′	7.34 t (8.1)	128.3	7.46 t (7.6)	128.5	7.50 t (7.5)	128.6
4′	7.50 t (8.1)	132.9	7.58 tt (7.6, 1.5)	133.2	7.61 t (7.5)	133.7
7′		165.5		165.5		165.8
3-OCH_3			3.57 s	52.0		

[a] Assignments were based on the ^{13}C NMR chemical shift values of isopimarane-type diterpenes obtained from *O. stamineus*. The C-18 resonates always at lowerfield than C-19.[5–8]

but comparison of the ^{13}C NMR spectra showed the presence of a signal for a cyano group (δ_C 117.8) in **6** and the disappearance of a carboxyl group (δ_C 176.8, C-2) in **4**. In the HMBC experiment, the carbon at δ_C 117.8 was correlated with the protons at δ_H 2.57 and 2.28 (H_2-1), suggesting the location of the cyano group to be at C-2. Thus, secoorthosiphol C was determined to have the structure formula **6**.

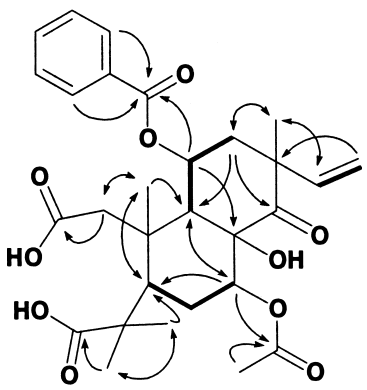


Figure 4. Partial structures (bold line) deduced by the COSY and HMQC spectra and significant HMBC correlations (arrow) for **4**.

The HRFABMS of orthosiphol R (**7**) showed the quasimolecular ion at m/z 657.2648 $(M+Na)^+$, consistent with the molecular formula $C_{36}H_{42}O_{10}$. The IR spectrum of **7** closely resembled that of orthosiphol L[7] (**13**) and showed absorptions of hydroxyl, ester carbonyl and phenyl groups. The ^{1}H and ^{13}C NMR spectra of **7** were also similar to those of **13** (Table 3), but analysis of the COSY and HMQC spectra indicated an upfield shift of H-2 (δ_H 4.45) and a downfield shift of H-3 (δ_H 5.03) compared to those of **13** (H-2, δ_H 5.47; H-3, δ_H 3.50). Thus, the hydroxyl and acetyl groups were assumed to be located at C-2 and C-3, respectively, which was confirmed by the HMBC spectrum. The stereochemistry of **7** was determined to be the same as **13**, i.e. all chair conformation in rings A–C, based on the ROESY correlations of H-2 with H-1, H-3, H_3-19 and H_3-20, of H-11 with H_3-20 and of H-5 with H-9. The orientation of the hydroxyl group at C-12 was concluded to be α based on the broad singlet nature of H-12 ($J_{1/2}$=4.3 Hz). From these data, orthosiphol R was concluded to have the structure formula **7**. This compound is the second example of isopimaran-type diterpene isolated from *O. stamineus* and possessing a hydroxyl group at C-12.

The molecular formula of orthosiphol S (**8**) was determined by HRFABMS to be $C_{34}H_{36}O_9$. The ^{1}H NMR spectrum of **8** was similar to that of orthosiphol N[7] (**15**) but was

Table 3. ^{1}H and ^{13}C NMR data for compounds **7–9** in $CDCl_3$ (*J* values in parentheses)

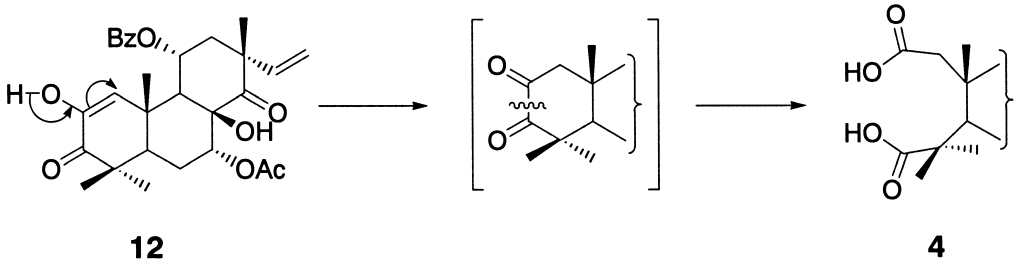
Position	**7**		**8**		**9**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4.90 d (3)	78.6	6.13 s	81.1	5.41 d (2.5)	73.7
2	4.45 br s	66.2		200.5	5.49 t (2.5)	67.5
3	5.03 d (3)	78.5	5.73 s	79.9	3.48 br s	78.1
4		37.2		40.6		38.6
5	2.39 dd (11.1, 4.5)	36.8	2.23 dd (11.0, 4.3)	40.9	2.66 dd (11.2, 4.0)	34.5
6	2.06 m	21.4	1.85 m	25.5	2.01 m	23.5
7	5.51 t (2.5)	71.2	4.35 s	68.1	4.21 br s	69.2
8		74.7		77.7		77.2
9	3.39 d (7.7)	41.9	1.94 s	51.0	2.99 d (6.5)	42.7
10		44.0		44.6		43.9
11	5.84 d (7.7)	73.2		205.9	5.60 d (6.5)	69.0
12	3.78 br s	77.1	2.85 d (17.7)	47.7	2.58 dd (15.2, 6.5)	39.0
			2.63 d (17.7)		2.25 dd (15.2)	
13		54.2		49.3		47.8
14		205.2		210.6		113.8
15	5.69 dd (17.5, 10.9)	137.6	5.48 dd (17.5, 10.5)	139.1	5.75 dd (17.7, 10.8)	141.9
16	5.04 d (17.5)	115.7	4.81 d (17.5)	117.0	4.87 d (17.6)	113.2
	4.94 d (10.9)		4.71 d (10.5)		4.69 d (10.8)	
17	1.29 s	21.9	1.22 s	25.4	1.26 s	28.1
18	0.87 s	27.8	1.20 s	23.1	1.16 s	28.6
19	1.09 s	22.5	1.14 s	25.2	1.03 s	22.1
20	1.59 s	16.6	1.70 s	18.8	1.45 s	17.3
1-OBz						
1′		129.4		130.2		130.3
2′,6′	7.81 d (7.0)	129.7	7.92 d (7.1)	130.0	7.49 d (7.6)	129.5
3′,5′	7.40 t (7.0)	128.2	7.33 t (7.1)	128.3	7.20 d (7.6)	128.3
4′	7.63 t (7.0)	133.3	7.48 t (7.1)	133.2	7.40 t (7.6)	132.7
7′		167.6		164.3		164.5
3-OBz						
1″				129.7		
2″,6″			7.99 d (8.3)	129.8		
3″,5″			7.35 t (8.3)	128.0		
4″			7.50 t (8.3)	132.6		
7″				167.7		
11-OBz						
1‴		129.5				130.1
2‴,6‴	7.52 d (7.0)	130.0			7.47 d (7.6)	129.4
3‴,5‴	7.16 t (7.0)	128.3			6.98 t (7.6)	127.6
4‴	7.50 t (7.0)	133.6			7.30 t (7.6)	132.0
CO		166.5				165.7
2-OAc						
CO*CH*$_3$					1.92 s	20.9
*C*OCH$_3$						170.1
3-OAc						
CO*CH*$_3$	1.39 s	20.2				
*C*OCH$_3$		170.6				
7-OAc						
CO*CH*$_3$	2.20 s	21.0				
*C*OCH$_3$		168.3				

characterized by two sharp singlets at δ_H 6.13 (H-1) and 5.73 (H-3), together with disappearance of signals due to an oxymethine proton and an acetyl methyl in **15**. On the other hand, the ^{13}C NMR spectra of **8** and **15** showed the difference corresponding to that in the ^{1}H NMR spectra with one additional ketone carbonyl carbon (δ_C 200.5) in **8** (Table 3). These data suggested that the acetoxy-substituted carbon (C-2) in **15** should be replaced by the ketone carbonyl carbon in **8**, which was confirmed by the HMBC correlations between the ketone carbonyl carbon and the protons at δ_H 6.13 (H-1) and 5.73 (H-3).

The ROESY correlations H_3-19/H_3-20 and H-5/H-9 indicated the stereochemistry of **8** to be the same as that of **15**.

The molecular formula of orthosiphol T (**9**) was determined from HRFABMS to be $C_{38}H_{46}O_{12}$. The ^{1}H and ^{13}C NMR spectra of **9** resembled those of orthosiphol A (**10**), but they were characterized by the lack of one of two acetyl groups. Analysis of the COSY and HMQC spectra indicated a high-field shift of H-7 (δ_H 4.21), compared to that of **10** (δ_H 5.43). Thus, orthosiphol T (**9**) was assumed to be 7-*O*-deacetyl-orthosiphol A, which was confirmed by the HMBC and ROESY spectra.

In this paper, we have reported nine novel diterpenes, norstaminolactone A (**1**), norstaminols B and C (**2** and **3**), secoorthosiphols A–C (**4–6**)[12] and orthosiphols R–T (**7–9**), together with nine known diterpenes, orthosiphols A (**10**), B (**11**), E (**12**), L–N (**13–15**) and P (**16**), and neoorthosiphol A (**17**) and norstaminone A (**18**). Among these, **1** is the first example of a biogenetically exclusive staminane-type



Scheme 2. Possible biogenetic pathway to secoorthosiphol A (**4**) from orthosiphol E (**12**).

diterpene having a nitrogen atom in a molecule, while **4**–**6** are the first examples of 2,3-secoisopimarane-type diterpenes and **6** is the first example of biogenetically unique and unusual isopimarane-type diterpene bearing a cyano group. These secoisopimarane-type and unusual staminane-type diterpenes seemed to be characteristic constituents in *O. stamineus* of Okinawa.

Staminane-type diterpenes are believed to be biosynthesized from its isopimarane precursor, through the migration of vinylic group from C-13 to C-12 with the possible involvement of cytochrome P-450.[7] The Baeyer–Villigar-type oxidation of staminane-type diterpenes followed by ketal formation may give norstaminolactone A (**1**). The intermediate species after Baeyer–Villigar oxidation might undergo oxidative decarboxylation followed by dihydroxylation of vinylic group and rearrangement to give norstaminol B (**2**). On the other hand, rotation around C_{11}–C_{12} of the dihydroxylated intermediate species and subsequent ketal formation gives norstaminol C (**3**) (Scheme 1). Similarly, secoorthosiphols A–C (**4**–**6**) may be produced through an oxidative cleavage of the bond between C-2 and C-3 of **12**, which was also isolated from the same extract (Scheme 2). The absolute configuration of neoorthosiphol A (**17**) has been established by Shibuya et al. by exciton chirality method.[9] By considering biogenetic point of view, all the isolated novel compounds might have the same absolute stereochemistry as **17**.

All the newly isolated compounds were tested for their antiproliferative activity towards highly liver metastatic murine colon 26-L5 carcinoma[13] and human HT-1080 fibrosarcoma[14] cell lines (Table 4).[15] They showed selective activity toward murine colon 26-L5 carcinoma cell line, with different potency in a dose-dependent manner. Among the newly isolated compounds, norstaminolactone A (**1**) showed the most potent activity with an IC_{50} value of 2.16 μg/mL.

Table 4. Antiproliferative activity of compounds **1**–**9** (IC_{50} values in μg/mL)

Compounds	Colon 26-L5 carcinoma	HT-1080 fibrosarcoma
1	2.16	27.9
2	38.8	>100
3	18.4	>100
4	63.5	>100
5	62.5	>100
6	45.7	>100
7	40.8	38.2
8	40.8	83.6
9	11.6	82.4
5-fluorouracil	0.015	0.48

ED_{50} values were calculated from the mean of data of four determinations.

3. Experimental

3.1. General experimental procedures

Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solutions. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. HRFABMS measurements were carried out on a JEOL JMS-700T spectrometer and glycerol was used as matrix. Column chromatography was performed with BW-820MH silica gel (Fuji Silysia, Aichi, Japan). Analytical and preparative TLC were carried out on precoated silica gel plates (Merck, 0.25 or 0.50 mm thickness).

3.2. Plant material

The aerial parts of cultivated *O. stamineus* Benth. were collected at Naha, Okinawa in November, 2000. A voucher sample (TMPW 20629) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

3.3. Extraction and isolation

Air-dried aerial parts of *O. stamineus* (1.8 kg) were extracted with MeOH (6 L, reflux, 3 h×3). The MeOH extract (162 g) was suspended in H_2O (1 L) and partitioned successively with hexane, $CHCl_3$, EtOAc and BuOH (each 1 L×3) to yield hexane (63 g), $CHCl_3$ (10 g), EtOAc (9 g), BuOH (18 g) and H_2O (62 g) fractions, respectively. The $CHCl_3$ fraction (10 g) was chromatographed with an EtOAc–hexane solvent system to give six fractions [fraction 1: EtOAc–hexane (1:4) eluate, 4.2 g; fraction 2: EtOAc–hexane (1:3) eluate, 678 mg; fraction 3: EtOAc–hexane (1:2) eluate, 468 mg; fraction 4: EtOAc–hexane (1:1) eluate, 474 mg; fraction 5: EtOAc–hexane (3:2) eluate, 313 mg; fraction 6: EtOAc–hexane (3:1) eluate, 3.25 g].

Fraction 2 (678 mg) was rechromatographed (1×35 cm) with hexane–EtOAc (2:1) to afford three subfractions (fraction 2-1, 90 mg; fraction 2-2, 310 mg; fraction 2-3, 220 mg). Subfraction 2-1 was separated by preparative

TLC with 2.5% MeOH–$CHCl_3$ to give norstaminols B (**2**, 3.7 mg) and C (**3**, 2.5 mg) and orthosiphol A (**10**, 10 mg). Subfraction 2-2 was subjected to rechromatography with 2.5% MeOH–$CHCl_3$ followed by purification with preparative TLC in 15% acetone–benzene to give orthosiphols B (**11**, 207 mg), E (**12**, 8 mg), M (**14**, 60 mg) and N (**15**, 50 mg), while subfraction 2-3 was separated by preparative TLC with 15% acetone–benzene and then with 2.5% MeOH–$CHCl_3$ to give **11** (20 mg), **14** (10 mg), **15** (8.0 mg) and secoorthosiphols B (**5**, 1.0 mg) and C (**6**, 2.5 mg).

Fraction 3 (468 mg) was rechromatographed (1×35 cm) with hexane–EtOAc (2:1) to afford three subfractions (fraction 3-1, 60 mg; fraction 3-2, 130 mg; fraction 3-3, 150 mg). Subfraction 3-1 was separated by preparative TLC with 2.5% MeOH–$CHCl_3$ to give **2** (6.1 mg), **3** (0.5 mg) and **11** (4.6 mg). Subfraction 2-2 or 2-3 was subjected to preparative TLC with 2.5% MeOH–$CHCl_3$ and then with 15% acetone–benzene to give orthosiphol L (**13**, 3.0 mg) and neoorthosiphol A (**17**, 40 mg) or orthosiphols R (**7**, 1.5 mg), T (**9**, 2.5 mg), **11** (3.0 mg), **13** (5.0 mg) and **15** (3.0 mg), respectively.

Fraction 4 (474 mg) was rechromatographed (1×35 cm) with 2.5% MeOH–$CHCl_3$ to afford three subfractions (fraction 4-1, 80 mg; fraction 4-2, 130 mg; fraction 4-3, 90 mg). Subfraction 4-1 was separated by preparative TLC with 2.5% MeOH–$CHCl_3$ to give norstaminolactone A (**1**, 2.0 mg) and **17** (40 mg). Subfraction 4-2 was purified by preparative TLC with 2.5% MeOH–$CHCl_3$ to give **17** (80 mg), while subfraction 4-3 was separated by preparative TLC with 15% acetone–benzene to give orthosiphol P (**16**, 3.0 mg), **7** (1.7 mg) and **18** (1.6 mg).

Fraction 5 (313 mg) was chromatographed (1×35 cm) with 2.5% MeOH–$CHCl_3$ to yield three subfractions (fraction 5-1, 95 mg; fraction 5-2, 56 mg; fraction 5-3, 36 mg). Each subfraction was subjected to preparative TLC with 2.5% MeOH–$CHCl_3$ to give **11** (5.0 mg), **17** (40 mg) and **18** (3.3 mg); **8** (15.2 mg); and **4** (15.2 mg), respectively.

3.3.1. Norstaminolactone A (1). Colorless amorphous solid, $[\alpha]_D^{25}=-18.3°$ (*c* 0.25, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 3250, 1800, 1740, 1610, 1600, 1450, 1420, 1370, 1280, 1110, 1050 cm^{-1}. HRFABMS 706.2831 [calcd for $C_{38}H_{44}O_{12}N$ $(M-H)^-$, 706.2864]. ^1H and ^{13}C NMR, see Table 1.

3.3.2. Norstaminol B (2). Colorless amorphous solid, $[\alpha]_D^{25}=-31.0°$ (*c* 0.37, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 1725, 1600, 1455, 1370, 1280, 1200–1240, 1110 cm^{-1}. HRFABMS 695.2701 [calcd for $C_{37}H_{43}O_{13}$ $(M-H)^+$, 695.2704]. ^1H and ^{13}C NMR, see Table 1.

3.3.3. Norstaminol C (3). Colorless amorphous solid, $[\alpha]_D^{25}=+115.1°$ (*c* 0.04, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3500, 1710, 1675, 1590, 1450, 1360, 1265, 1110, 1065 cm^{-1}. HRFABMS 579.2161 [calcd for $C_{30}H_{36}O_{10}Na$ $(M+H)^-$, 579.2206]. ^1H and ^{13}C NMR, see Table 1.

3.3.4. Secoorthosiphol A (4). Colorless amorphous solid, $[\alpha]_D^{25}=-155.6°$ (*c* 0.5, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 1710, 1600, 1420, 1370, 1280, 1110, 1050 cm^{-1}. HRFABMS 543.2272 [calcd for $C_{29}H_{35}O_{10}$ $(M-H)^-$, 543.2230]. ^1H and ^{13}C NMR, see Table 2.

3.3.5. Secoorthosiphol B (5). Colorless amorphous solid, $[\alpha]_D^{25}=-340.6°$ (*c* 0.05, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 1710, 1600, 1420, 1370, 1280, 1110, 1050 cm^{-1}. HRFABMS 557.2385 [calcd for $C_{30}H_{37}O_{10}$ $(M-H)^-$, 557.2387]. ^1H and ^{13}C NMR, see Table 2.

3.3.6. Secoorthosiphol C (6). Colorless amorphous solid, $[\alpha]_D^{25}=-99.6°$ (*c* 0.05, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 2250, 1710, 1600, 1420, 1370, 1280, 1110, 1050 cm^{-1}. HRFABMS 524.2281 [calcd for $C_{29}H_{38}NO_8$ $(M-H)^-$, 524.2284]. ^1H and ^{13}C NMR, see Table 2.

3.3.7. Orthosiphol R (7). Colorless amorphous solid, $[\alpha]_D^{25}=-151.8°$ (*c* 0.05, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 3450, 1720, 1455, 1600, 1370, 1280, 1090, 1040 cm^{-1}. HRFABMS 657.2648 [calcd for $C_{36}H_{42}O_{10}Na$ $(M+Na)^+$, 657.2676]. ^1H and ^{13}C NMR, see Table 3.

3.3.8. Orthosiphol S (8). Colorless amorphous solid, $[\alpha]_D^{25}=-75.5°$ (*c* 0.1, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3600, 2585, 1720, 1655, 1600, 1520, 1480, 1425, 1210, 1115, 1040, 930 cm^{-1}. HRFABMS 699.2235 [calcd for $C_{34}H_{36}O_9Na$ $(M+Na)^+$, 699.2257]. ^1H and ^{13}C NMR, see Table 3.

3.3.9. Orthosiphol T (9). Colorless amorphous solid, $[\alpha]_D^{25}=-99.6°$ (*c* 0.05, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3550, 3400, 1720, 1600, 1455, 1370, 1315, 1280, 1110, 1070, 1045 cm^{-1}. HRFABMS 693.2874 [calcd for $C_{38}H_{45}O_{12}$ $(M+H)^+$, 693.2911]. ^1H and ^{13}C NMR, see Table 3.

3.3.10. Conversion of secoorthosiphol A (4) into secoorthosiphol A dimethylester (19). Ethereal diazomethane (1 mL) was added to a **4** (2 mg) in a 1.5 mL glass vial and reacted for 10 min with occasional swirling. The diethyl ether and unreacted diazomethane were allowed to evaporate at room temperature to give dimethoxysecoorthosiphol A (**19**). Colorless amorphous solid, FABMS *m/z* 571 $C_{31}H_{39}O_{10}$ $(M-H)^-$. ^1H NMR ($CDCl_3$, 400 MHz): 8.03 (2H, dd, *J*=7.5, 1.5 Hz, H-2′,6′), 7.58 (1H, tt, *J*=7.5, 1.5 Hz, H-4′), 7.46 (2H, t, *J*=7.5 Hz, H-3′,5′), 5.95 (1H, dd, *J*=17.7, 10.8 Hz, H-15), 5.60 (1H, dd, *J*=7.3 Hz, H-11), 5.30 (1H, t, *J*=2.8 Hz, H-7), 5.15 (1H, d, *J*=10.8 Hz, H_E-16), 4.98 (1H, d, *J*=17.7 Hz, H_Z-16), 3.77 (1H, dd, *J*=7.3, 1.9 Hz, H-9), 3.53 (3H, s, 3-OCH_3), 3.06 (1H, dd, *J*=14.7, 2.8 Hz, H-5), 2.96 (3H, s, 2-OCH_3), 2.54 (1H, dd, *J*=15.6, 7.3 Hz, H-12_{ax}), 2.21 (2H, d, *J*=8.7 Hz, H_2-1), 2.09 (3H, s, 7-$OCOCH_3$), 2.08 (1H, m, H_2-6), 2.06 (1H, m, H_{eq}-12), 1.18 (3H, s, H_3-19), 1.18 (3H, s, H_3-20), 1.17 (3H, s, H_3-17), 1.17 (3H, s, H_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): 208.9 (s, C-14), 179.0 (s, C-3), 170.9 (s, C-2), 169.4 (s, 7-*O*C*O*CH_3), 165.5 (s, 11-*O*C*O*Ph), 140.5 (d, C-15), 133.2 (d, C-4′), 130.3 (d, C-1′), 129.8 (d, C-2′,6′), 128.4 (d, C-3′,5′), 114.5 (t, C-16), 75.1 (s, C-8), 71.0 (d, C-7), 69.1 (d, C-11), 51.8 (q, 3-OCH_3), 50.0 (q, 2-OCH_3), 47.8 (s, C-13), 45.4 (s, C-10), 41.7 (d, C-9), 41.4 (s, C-4), 41.2 (d, C-5), 40.1 (d, C-1), 39.7 (t, C-12), 27.8 (q, C-18), 25.6 (q, C-17), 23.7 (q, C-19), 23.5 (t, C-6), 21.1 (q, 7-OCO*C*H_3), 20.1 (q, C-20).

3.4. Antiproliferative assay

The antiproliferative assay was carried out, using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium (MTT) method,[16] by the same procedure as reported previously.[7] 5-Fluorouracil, a clinically used drug,[17] was taken as a positive control. The cultured cells were treated with the isolated compounds at five different concentrations (1–100 μg/mL), while for the positive control, five different concentrations ranging from 1 to 0.001 μg/mL were used. The assay was performed in quadruplicate and results are expressed by IC_{50} values (μg/mL).

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