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Torrubiellutins A–C, from insect pathogenic fungus *Torrubiella luteorostrata* BCC 12904

Pattama Pittayakhajonwut *, Atit Usuwan, Chakapong Intaraudom, Punsa Khoyaiklang, Sumalee Supothina

National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Paholyothin Road, Klong Luang, Pathumthani 12120 Thailand

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ABSTRACT

Investigation of the insect pathogenic fungus *Torrubiella luteorostrata* led to the isolation of three new macrocyclic torrubiellutins A–C (**1**–**3**) and the known pyrone diterpene **4**. Structures were elucidated by spectroscopic data including 1D, 2D NMR, and MS spectral data. The absolute stereochemistry was determined by chemical means using Mosher reactions and Marfey's reagent, together with NOESY spectral data. Torrubiellutin C showed biological activities against KB, MCF-7, NCI-H187, and Vero cell lines with IC_{50} varying from 0.78 to 4.36 μg/mL, while compound **4** exhibited antimalaria and anti-inflammatory activity with IC_{50} values of 3.49 and 1.21 μg/mL, respectively.

1. Introduction

Insect fungi have consistently been shown to be a good source of bioactive metabolites.[1] Several compounds isolated from insect fungi in our BIOTEC Culture Collection were described together with their biological activities. As a part of our on-going search for bioactive substances from microorganisms, the crude extracts of the insect fungus *Torrubiella luteorostrata* BCC 12904 exhibited biological activity against human breast cancer (MCF-7) and human epidermoid carcinoma (KB) with IC_{50} values of 1.03–10.37 and 1.27–15.52 μg/mL, respectively. Further investigation led to the isolation of new macrocyclic torrubiellutins A–C (**1**–**3**), and the pyrone diterpene **4**. Biological activity of isolated compounds was also evaluated.

2. Results and discussion

The extract obtained from culture broth was subjected to Sephadex LH20 chromatography followed by either silica column or reversed-phase HPLC to afford pure torrubiellutins A (**1**) and B (**2**). In addition, purification of the mycelium extract yielded zeorin [$C_{30}H_{52}O_2$, 215–217 °C],[2] torrubiellutins B (**2**) and C (**3**), and pyrone diterpene (**4**).

Compound **1**, obtained as a colorless solid, the molecular formula of which was determined as $C_{26}H_{37}NO_5$ based on HRESIMS showing *m*/*z* peak at 466.2577 $[M+Na]^+$, Δ +1.3 mmu. ^{13}C NMR spectrum together with DEPT-135 spectral data gave 24 signals of 6 methyl, 1 methylene, 13 methine, and 4 quaternary carbons. Two aromatic methine signals at δ_C 128.2 and 129.3 contained two carbons each. COSY and HMBC spectra gave, respectively, ^1H–^1H and ^1H–^{13}C correlations as shown in Table 1. The spectral information gave the partial structures from C-1 to C-11 and from C-14 to aromatic ring. The correlation of methyl at δ_H 2.79 to the C-1 in HMBC spectrum indicated an amide linkage. In addition, the methine at H-11 (δ_H 5.05), suggesting a connection to an oxygen, together with the remaining carbon at δ_C 170.4 (C-13) led to the chemical structure of compound **1**, named as torrubiellutin A. Torrubiellutin A (**1**) is a macrocyclic compound containing an amino acid unit. The configuration of amino acid, *N*-methyl-phenylalanine (*N*MePhe), was determined by acid hydrolysis of **1** with 6 N HCl and then derivatization with Marfey's reagent (FDAA).[3,4] The sample was co-injected with L-FDAA derivatives of standard *N*MePhe (D- and L-forms) and analyzed by reversed-phase HPLC. The result indicated the L-form of *N*MePhe. The molecule has stereogenic centers at C-4, C-5, C-8, C-9, C-10, and C-11. The absolute configurations at C-5 and C-9 were assigned by employing the Mosher method.[5] Treatment of **1** with *R*- and *S*-MTPACl afforded compounds **5** and **6**, respectively. The differences in chemical shift values ($\Delta\delta_{R-S}$) of diester derivatives **5b** and **6b** (given in Fig. 1) were

* Corresponding author. Tel.: +66 25646700x3559; fax: +66 25646632.
E-mail address: pattamap@biotec.or.th (P. Pittayakhajonwut).

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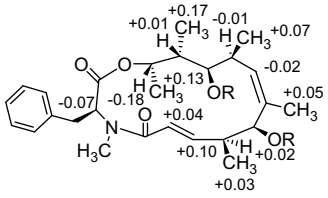
Table 1
1D and 2D NMR assignment of torrubiellutin A in acetone-d_6

No.	δ_H (int., mult., J in Hz)[a]	δ_C[b]	COSY	HMBC (H→C#)
1	—	166.5	—	—
2	6.28 (1H, d, 15.0)	119.2	—	1, 4
3	7.07 (1H, dd, 15.0, 10.3)	149.1	—	1
4	2.68–2.71 (1H, m)	39.5	H-5	18
5	3.94 (1H, d, 5.1)	77.7	—	6, 7
6	—	136.0	—	—
7	5.66 (1H, dd, 11.0, <1)	122.8	—	5, 19
8	2.58–2.62 (1H, m)	34.2	H-7	—
9	3.15 (1H, ddd, 11.0, 9.3, 1.1)	76.4	—	7
10	1.56–1.58 (1H, m)	41.3	—	—
11	5.05 (1H, dq, 6.5, 3.7)	72.6	—	—
13	—	170.4	—	—
14	3.74 (1H, dd, 10.5, 4.4)	67.7	—	—
16	3.24 (1H, dd, 13.8, 4.4), 3.33 (1H, dd, 13.8, 10.5)	35.2	H-14	1′, 2′, 6′
17	2.79 (3H, s)	38.2	—	1
18	1.18 (3H, d, 7.0)	18.8	H-4	3, 4, 5
19	1.56 (3H, d, 0.9)	14.5	—	5, 6, 7
20	1.01 (3H, d, 6.8)	17.8	H-8	7, 8, 9
21	0.69 (3H, d, 6.8)	9.3	H-10	9, 10, 11
22	1.10 (3H, d, 6.5)	12.6	H-11	10, 11
1′	—	139.6	—	—
2′/6′	7.19–7.30 (2H, m)	129.3×2	H-3′, H-5′	4′, 16
3′/5′	7.19–7.30 (2H, m)	128.1×2	H-2′, H-4′, H-6′	1′
4′	7.19–7.30 (1H, m)	126.1	—	—

[a] Recorded at 500 MHz.
[b] Recorded at 125 MHz.

calculated in order to assign the absolute configurations at C-5 and C-9 as *S*- and *R*-, respectively. Due to the limited amount of compound **1**, the absolute configurations of C-4, C-8, C-10, and C-11 were determined by NOESY spectral data and coupling constant values (J). In the presence of D_2O, H-5 and H-9 appeared as a singlet and double doublet (J=11.0, <1 Hz), respectively. The former indicated no coupling between H-4 and H-5. In addition, the conformation of H-4 and H-5 was restricted by the configuration of two olefins at C-2 and C-6. The olefinic proton at H-2 coupled to H-3 with a coupling constant of 15.0 Hz indicated a *trans*-configuration and the cross-peak correlation showing in NOESY spectrum between H-7 and H_3-19 indicated a *cis*-configuration of the olefin at C-6. Moreover, the NOESY spectral data displayed cross-peak correlations from H-2 to H-4; H-3 to H-10; H-4 to H_3-19, suggesting *R*-configuration at C-4. The double doublet of H-9 (J=11.0, <1 Hz) was changed to a doublet with a coupling constant of 11.0 Hz after irradiation at H-8. Thus, it was implied that the coupling constant between H-9 and H-10 is 11.0 Hz and that between H-8 and H-9 is small (J<1 Hz). Together with the cross-peak in NOESY spectrum, which correlated H-9 to H_3-21; H-8 to H_3-19 and H_3-21, this indicated an *anti*-relationship between H-9 and H-10 and a *syn*-relationship between H-8 and H-9. The absolute configurations at C-8 and C-10 can therefore be assigned as *S*- and *R*-, respectively. The coupling constant agreed with the dihedral angle of 95.2° between H-8 and H-9 as calculated from the most favorable conformation shown in Figure 2. The absolute configuration at C-11 was assigned as *S*- based on the evidence from NOESY correlation between H-3 and H-10 and the coupling constant of 3.7 Hz between H-10 and H-11. The dihedral angle of −85.8° was also calculated based on the conformation shown in Figure 2. Therefore, six stereogenic centers can now be assigned as 4*R*, 5*S*, 8*S*, 9*R*, 10*R*, and 11*S*, respectively. The chemical structure of torrubiellutin A (**1**) can be depicted as shown in Figure 3.



5b) $R^1 = R^2$ = *S*-MTPA
6b) $R^1 = R^2$ = *R*-MTPA

Figure 1. $\Delta\delta$ values ($\Delta\delta=\delta_R-\delta_S$) obtained from **5b** and **6b**.

Compound **2**, a colorless solid, gave similar ^{1}H NMR and ^{13}C NMR spectral data to compound **1**. The ^{1}H NMR spectrum displayed two additional methyl protons at δ_H 2.11 and 2.16, which correlated in the HMBC spectrum to the carbonyl esters at δ_C 170.26 and 169.20, respectively. The data indicated the presence of two additional acetyl groups in the molecule. Also the lower field shift of two methine protons at δ_H 4.72 (H-9) and 5.07 (H-5) ($\Delta\delta$=1.57 and 1.13 ppm, respectively) suggested the two acetyl groups being substituted at H-9 and H-5, respectively. HRESIMS confirmed the molecular formula $C_{30}H_{41}O_7N$ showing m/z peak at 550.2784 $[M+Na]^+$, Δ +0.9 mmu. The methine protons at H-5 and H-9 also appeared as a singlet and a double doublet (J=11.5 and 2.4 Hz), respectively. Irradiation at H-10 resulted in a coupling constant of 2.4 Hz, suggesting a *gauche* conformation between H-8 and H-9. The result indicated an angle distortion of H-8 and H-9 in order to avoid steric effect from the acetyl group. The cross-peak correlation from H-9 to H_3-20 was also observed in NOESY spectrum. Therefore, the chemical structure of compound **2**, named as torrubiellutin B, was established as shown in Figure 3. Although, torrubiellutin B was isolated as a major product, it could not be obtained as a single crystal for X-ray crystallographic study.

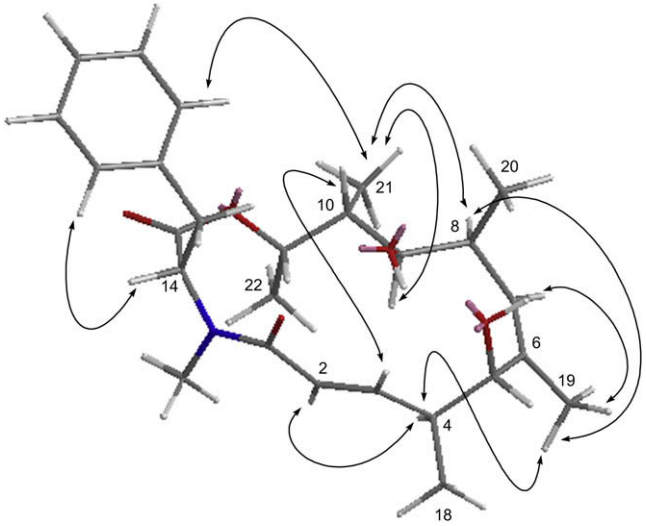
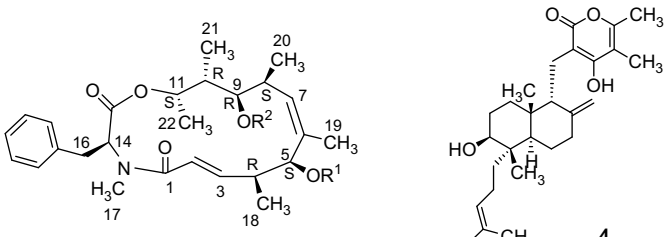


Figure 2. 3D structural conformation of torrubiellutin A generated by MM2 force field calculations for energy minimization from modeling program Chem3D Ultra 9.0 with the observed NOE correlations (arrows).

Compound **3**, named as torrubiellutin C, was obtained as a colorless solid. ^{1}H NMR and ^{13}C NMR spectral data are similar to those of **1** except for an extra methyl group at δ_H 2.17, which correlated in HMBC to the carbonyl ester at δ_C 171.27. This indicated the presence of an acetyl group in the molecule. HRESIMS determined the molecular formula $C_{28}H_{39}O_6N$, showing m/z peak at 508.2669 $[M+Na]^+$, Δ −0.6 mmu. HMBC spectral data showed correlations from H-9 to C-6; H_3-18 to C-3, C-4 and C-5; H_3-19 to H-5 and H-6; H_3-20 to H-7 and H-8. The lower field shift of H-9 at δ_H 4.77 ($\Delta\delta$=1.62 ppm) indicated the presence of an acetyl group at C-9. Coupling patterns of H-5 and H-9 were also similar to those of compound **2**. H-5 appeared as a singlet and H-9 appeared as



1) $R^1 = R^2 = H$

2) $R^1 = R^2 = COCH_3$

3) $R^1 = H$, $R^2 = COCH_3$

5a) R^1 = *S*-MTPA, $R^2 = H$

5b) $R^1 = R^2$ = *S*-MTPA

6a) R^1 = *R*-MTPA, $R^2 = H$

6b) $R^1 = R^2$ = *R*-MTPA

4

Figure 3. Chemical structures of compounds **1**–**5**.

double doublet with coupling constants of 11.5 and 2.4 Hz. NOESY spectral data provided the same information as in compound **2**. Thus, the chemical structure of torrubiellutin C can be depicted as shown in Figure 3.

Compound **4** was obtained as colorless solid, $[\alpha]_D^{29}$ −62.8 (*c* 0.10, MeOH).[6] ^{1}H NMR and ^{13}C NMR spectra recorded in mixture of $CDCl_3$ and CD_3OD were consistent with those given in the patent,[6] known as BR-050. Those data in DMSO-d_6 were also reported in the experimental section (4.2.4).

Torrubiellutins A and B were inactive against KB, MCF-7, NCI-H187 cell lines and malaria, *Plasmodium falciparum* K1 strain, but showed cytotoxicity against Vero cell line with IC_{50} values of 12.33 and 43.66 μg/mL, respectively. While torrubiellutin C, having one acetyl group at C-9, exhibited strong cytotoxicity against KB, MCF-7, NCI-H187, and Vero cell lines with IC_{50} varying from 0.78 to 4.36 μg/mL (see Table 2). Compound **4** also displayed antimalarial activity with an IC_{50} value of 3.49 μg/mL and also exhibited strong anti-inflammatory against COX-2 with IC_{50} value of 1.21 μg/mL, while the inflammatory activity against COX-1 was inactive. This is the first report of antimalarial and anti-inflammatory activity of BR-050. Compound **4** was reported to inhibit bone resorption and is useful for diseases with abnormal bone metabolism.[6] In addition, the diterpenoids with a pyrone moiety, such as sesquicillin,[7] pycnophorin[8] exhibited various biological activities including insecticidal, anticancer, antihypertensive, anti-inflammatory, antibacterial activities, etc.

Table 2
Biological activity of compounds **1**–**4**

Compound	Cytotoxicity (IC_{50}, μg/mL)				Antimalarial[c] (IC_{50}, μg/mL)	Anti-inflammatory[d] (IC_{50}, μg/mL)
	MCF-7[a]	KB[a]	NCI-H187[a]	Vero[b]		
1	Inactive	Inactive	Inactive	12.33	Inactive	nt
2	Inactive	Inactive	Inactive	43.62	Inactive	nt
3	0.78	1.30	1.32	4.36	Inactive	nt
4	6.17	0.85	2.58	0.69	3.49	COX-1=inactive, COX-2=1.21

nt=not tested.
[a] IC_{50}>20 μg/mL was inactive.
[b] IC_{50}>50 μg/mL was inactive.
[c] IC_{50}>20 μg/mL was inactive.
[d] IC_{50}>10 μg/mL was inactive.

3. Conclusion

Three macrocyclic torrubiellutins A–C (**1**–**3**) together with two known pyrone diterpene (**4**) and zeorin have been isolated from the insect pathogenic fungus *T. luteorostrata* BCC 12904. Torrubiellutin C exhibited cytotoxicity against KB, MCF-7, NCI-H187, and Vero cell lines with IC_{50} varying from 0.78 to 4.36 μg/mL, while torrubiellutins A and B were inactive against cancerous cell lines (KB, MCF-7, NCI-H187) but showed weak cytotoxicity against Vero cell line with IC_{50} values of 12.33 and 43.62 μg/mL, respectively. Compound **4** displayed antimalaria and anti-inflammatory activity against COX-2 with IC_{50} values of 3.49 and 1.21 μg/mL, respectively.

4. Experimental

4.1. General experimental procedures

Mps were determined on an Electrothermal IA9100 digital melting point apparatus and are uncorrected. UV spectra were recorded on a Cary 1E UV–visible spectrophotometer in 95% EtOH. IR spectra were taken on a Bruker VECTOR 22 FT-IR spectrometer. Specific rotations were measured in either 95% EtOH or MeOH using a JASCO P-1030 polarimeter. NMR spectra, including COSY, NOESY, DEPT, HMQC, and HMBC experiments, were recorded on a Bruker AV500D NMR spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz). High resolution ESIMS were performed on a Micromass LCT mass spectrometer; accurate mass was determined by lock mass calibration. High performance liquid chromatography (HPLC) was performed on a Dionex, UltiMate 3000. Column Chromatography was performed using Merck silica gel (230–400 mesh ASTM).

4.2. Fungus material, extraction, and isolation

T. luteorostrata Zimm. was isolated from a scale insect *Homoptera* sp. from the underside of an unidentified leaf collected at Doi Inthanon National Park, Chiang Mai, Thailand. The fungus was identified by Dr. N.L. Hywel-Jones and registered at BIOTEC Culture Collection (BCC) as BCC 12904. The fungus was grown on a surface at 25 °C in 20×1 L Erlenmeyer flasks, containing 250 mL medium, which consists of 3% sucrose, 2% malt extract, 0.2% bacto-peptone, 0.1% yeast extract, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4. After 26 days, the mycelium was separated by filtration and culture broth was extracted thrice with equal volume of EtOAc. A gum (0.47 g) obtained from the culture broth was subjected to Sephadex LH20 column chromatography, eluted with 100% MeOH, to give three fractions. Each fraction was further purified by either silica column, using EtOAc and hexane (3:2) as eluent, or reverse-phase HPLC, using LiChroCART 250-10 LiChrospher100 RP-18 column eluted with 55% CH_3CN in water, to yield torrubiellutin B (**2**, 62.5 mg) and torrubiellutin A (**1**, 5.9 mg). Mycelium was macerated in MeOH (two days) followed by CH_2Cl_2 (two days). The solvents were then combined, evaporated, and extracted with EtOAc (×3). A gum (5.63 g) was obtained and separation was conducted by using Sephadex LH20 column chromatography (eluted with 100% MeOH) followed by a silica column (eluted with EtOAc and hexane; 2:3) to give five fractions. The first and second fractions were further purified by another silica column using EtOAc/hexane (2:3) as eluent to yield zeorin (83.1 mg). Torrubiellutin B (**2**, 0.23 g) was obtained from the third fraction. The fourth fraction (0.45 g) was purified by a Sephadex LH20 column (eluted with 100% MeOH) followed by a silica column (eluted with EtOAc and hexane; 2:3), to provide torrubiellutins B (**2**, 0.18 g) and C (**3**, 13.1 mg), respectively. The fifth fraction gave a colorless solid of compound **4** (0.30 g).

*4.2.1. Torrubiellutin A (**1**)*

Colorless solid; mp 96 °C; $[\alpha]_D^{27}$ −103.7 (*c* 0.1255, EtOH); IR ν_{max} ($CHCl_3$): 3397, 2970, 2927, 1738, 1652, 1601, 1487, 1454, 1410, 1351,

1277, 1204, 1088, 991, 754, 702 cm^{-1}; UV λ_{max}^{MeOH} (log ε): 215.7 (4.03) nm; ^1H and ^{13}C NMR data in acetone-d_6 given in Table 1; HRESIMS: calcd for $C_{26}H_{37}NO_5Na$: 466.2564; found: m/z 466.2577 $[M+Na]^+$.

*4.2.2. Torrubiellutin B (**2**)*

Colorless solid; mp 107 °C; $[\alpha]_D^{25}$ −132.3 (*c* 0.1165, EtOH); IR ν_{max} (KBr): 3468, 2974, 2934, 1742, 1658, 1618, 1453, 1375, 1239, 1086, 1021, 930, 752, 702 cm^{-1}; UV λ_{max}^{MeOH} (log ε): 216.6 (4.37), 252.8 (3.97) nm; ^1H and ^{13}C NMR data in acetone-d_6 given in Table 3; HRESIMS: calcd for $C_{30}H_{41}NO_7Na$: 550.2775; found: m/z 550.2784 $[M+Na]^+$.

*4.2.3. Torrubiellutin C (**3**)*

Colorless solid; mp 79–80 °C; $[\alpha]_D^{26}$ −29.3 (*c* 0.1235, EtOH); IR ν_{max} (KBr): 3444, 2926, 1739, 1655, 1608, 1454, 1357, 1240, 1083, 1015, 979, 752, 702 cm^{-1}; UV λ_{max}^{EtOH} (log ε): 217.7 (4.12) nm; ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 3; HRESIMS: calcd for $C_{28}H_{39}NO_6Na$: 508.2675; found: m/z 508.2669 $[M+Na]^+$.

*4.2.4. BR-050 (**4**)*

Colorless solid; mp 171.6–172.5; $C_{27}H_{40}O_4$; ^1H NMR (500 MHz, DMSO-d_6) δ 0.67 (s, 3H), 0.86 (s, 3H), 1.09–1.14 (2H, m), 1.23 (dq, 1H, *J*=12.9, 4.4 Hz), 1.45 (dt, 1H, *J*=8.6, <1 Hz), 1.50–1.58 (m, 3H), 1.60 (s, 3H), 1.63 (dd, 1H, *J*=11.8, 2.4 Hz), 1.65 (s, 3H), 1.71 (td, 1H, *J*=11.5, 4.3 Hz), 1.83 (s, 3H), 1.89–1.94 (m, 2H), 1.98 (dt, 1H, *J*=11.6, <1 Hz), 2.05 (dd, 1H, *J*=11.6, 4.3 Hz), 2.11 (s, 3H), 2.35 (ddd, 1H, *J*=11.6, 5.4, 5.4 Hz), 2.44 (dd, 1H, *J*=13.2, 4.3 Hz), 2.68 (dd, 1H, *J*=13.2, 12.4 Hz), 3.35 (m, interfered with water in DMSO-d_6, 1H), 4.13 (d, 1H, *J*<1 Hz), 4.32 (d, 1H, *J*=5.1 Hz, O*H*), 4.44 (t, 1H, *J*<1 Hz), 5.11 (t, 1H, *J*=7.1 Hz), 10.06 (s, 1H, O*H*); ^{13}C NMR (125 MHz, DMSO-d_6) δ 10.8 (*C*H_3), 17.5 (*C*H_3), 17.9 (*C*H_3), 18.0 (*C*H_3), 21.8 (*C*H_2), 21.9 (*C*H_2), 23.0 (*C*H_2), 23.5 (*C*H_3), 26.0 (*C*H_3), 28.0 (*C*H_2), 31.1 (*C*), 34.4 (*C*), 37.7 (*C*), 37.8 (*C*), 38.6 (*C*), 41.1 (*C*), 55.1 (*C*), 72.2 (*C*), 102.5 (*C*), 106.8 (*C*), 109.6 (*C*), 125.7 (*C*), 130.5 (*C*), 149.3 (*C*), 155.1 (*C*), 165.6 (*C*), 165.0 (*C*); ESIMS m/z: 451.28 $[M+Na]^+$.

4.3. Determination of absolute stereochemistry

*4.3.1. Hydrolysis of torrubiellutin A (**1**) with 6 N HCl*

Torrubiellutin A (**1**, 2.0 mg) was heated at 110 °C with 6 N HCl (500 mL) for 15 h. The reaction was evaporated to dryness under vacuum and the resulting material was derivatized with Marfey's reagent.

4.3.2. Derivatization of amino acid with Marfey's (FDAA) reagent and HPLC analysis

The crude product obtained by following the procedure described in Section 4.3.1 was allowed to react with 50 μL L-FDAA (1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide) in acetone (10 mg/1 mL) and 1 M $NaHCO_3$ (100 μL). The mixture was warmed at 60 °C for 15 min. After cooling to room temperature, the mixture was neutralized with 2 N HCl (50 μL). The solution was diluted with 50% CH_3CN (100 μL) and subjected to HPLC analysis (15 μL injection).

The L-FDAA derivatives of *N*MePhe (D- and L-forms) were also prepared as previously described. Standards *N*MePhe was prepared by stirring Fmoc-*N*MePhe (20 mg) in piperidine and DMF for 30 min at rt. The mixture was evaporated and water (1.5 mL) was added. The solution was washed twice with EtOAc. Then the water was evaporated to obtain *N*MePhe.

The FDAA derivatives of hydrolysate of **1** and standards *N*MePhe (D- and L-forms) were analyzed by HPLC using Nova-Pak C_{18} column (4 μm, 3.9×150 mm^2) with 30% CH_3CN in water containing 0.05% formic acid, flow rate 0.6 mL/min, 35 °C and detected at 340 nm.

The sample was also co-injected with L-FDAA derivatives of *N*MePhe (D- and L-forms). The retention times of FDAA derivatives of *N*Me-L-Phe and *N*Me-D-Phe were 19.71 and 21.09 min, respectively.

*4.3.3. Preparation of MTPA esters of compound **1***

To a solution of torrubiellutin A (**1**, 1.08 mg) in pyridine (100 μL) and CH_2Cl_2 (300 μL) was added (−)-MTPACl (40 μL). The solution was left at rt for four days. After removal of solvent, the mixture was

Table 3
^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) assignments of torrubiellutins B and C

No.	**2** (In acetone-d_6)		**3** (In $CDCl_3$)	
	δ_H (int., mult., *J* in Hz)[a]	δ_C[b]	δ_H (int., mult., *J* in Hz)[a]	δ_C[b]
1	—	166.1	—	166.6
2	6.40 (1H, d, 15.1)	120.2	6.08 (1H, d, 15.1)	119.1
3	7.08 (1H, dd, 15.1, 10.3)	147.2	7.17 (1H, dd, 15.1, 10.2)	149.0
4	2.87–2.92 (1H, m)	38.0	2.60–2.64 (1H, m)	39.8
5	5.07 (1H, s)	78.6	4.01 (1H, s)	78.3
6	—	133.3	—	136.9
7	5.25 (1H, dd, 10.9, 1.3)	122.6	5.47 (1H, d, 10.8)	122.6
8	2.74–2.78 (1H, m)	33.4	2.67–2.73 (1H, m)	33.7
9	4.72 (1H, dd, 11.5, 2.4)	76.8	4.77 (1H, dd, 11.5, 2.4)	77.0
10	1.76–1.79 (1H, m)	39.7	1.72–1.76 (1H, m)	39.8
11	4.61 (1H, dq, 6.5, 3.5)	71.3	4.70 (1H, dq, 6.5, 3.5)	72.1
13	—	170.0	—	170.2
14	3.80 (1H, dd, 10.4, 4.6)	67.0	3.54 (1H, dd, 9.5, 5.3)	67.9
16	3.26 (1H, dd, 13.7, 4.6), 3.34 (1H, dd, 13.7, 10.4)	35.1	3.36–3.40 (2H, m)	35.2
17	2.81 (3H, s)	38.0	2.74 (3H, s)	38.7
18	1.10 (3H, d, 7.0)	18.2	1.29 (3H, d, 7.0)	19.1
19	1.66 (3H, d, 1.0)	14.3	1.58 (3H, d, 0.4)	15.3
20	0.79 (3H, d, 6.79)	16.9	0.90 (3H, d, 6.8)	17.7
21	0.76 (3H, d, 6.8)	9.0	0.72 (3H, d, 6.9)	9.7
22	1.05 (3H, d, 6.5)	13.0	1.12 (3H, d, 6.5)	13.6
1′	—	139.4	—	139.2
2′/6′	7.20–7.31 (2H, m)	129.3×2	7.20–7.25 (2H, m)	128.4×2
3′/5′	7.20–7.31 (2H, m)	128.3×2	7.25–7.35 (2H, m)	128.5×2
4′	7.20–7.31 (1H, m)	126.2	7.20–7.25 (1H, m)	126.4
5-$OCOCH_3$	2.16 (3H, s)	19.95/169.2	—	
9-$OCOCH_3$	2.11 (3H, s)	19.90/170.3	2.17 (3H, s)	21.0/171.3

purified by HPLC, using SunFire Prep C_{18} OBD column (10 μm, 19×250 mm^2) and eluting with a gradient system varied from 30% to 70% CH_3CN in water over 20 min and then from 70% to 100% CH_3CN in water over 10 min, to afford *S*-MTPA esters **5a** (0.60 mg) and **5b** (0.23 mg). Similarly, compound **1** (0.69 mg) was reacted with (+)-MTPACl and after purification by HPLC, the mixture obtained *R*-MTPA esters **6a** (0.40 mg) and **6b** (0.22 mg).

4.4. Biological tests

Torrubiellutins A–C (**1**–**3**) and compound **4** were tested for cytotoxicity against KB, MCF-7, NCI-H187, Vero cell lines, and antimalarial activity against *Plasmodium falciparum* K1 strain (multi-drug resistant strain). Only compound **4** was evaluated for anti-inflammatory activity against COX-1 and COX-2. Cytotoxicity against cancer cell lines, including KB (human epidermoid carcinoma, ATCC CCL-17), MCF-7 (human breast cancer, ATCC HTB-22), and NCI-H187 (human small cell lung cancer, ATCC CRL-5804) were performed using the resazurin microplate assay,[9] and against Vero cell (African green monkey kidney fibroblasts, ATCC CCL-81) were performed using the green fluorescent protein microplate assay (GFPMA).[10] Ellipticine and doxorubicin were used as references for the cytotoxicity assay. Ellipticine displayed cytotoxicity against MCF-7, KB, NCI-H187, and Vero cell lines with IC_{50} values of 0.57, 0.45, 0.18, and 0.44–0.46 μg/mL, respectively. Doxorubicin showed biological activities against MCF-7, KB, and NCI-H187 with IC_{50} values of 0.19, 0.04–0.11, and 0.03 μg/mL, respectively. Antimalarial activity was performed using the microculture radio isotope technique described by Desjardins et al.[11] Dihydroartemisinin was used as reference and showed antimalarial activity with IC_{50} value of 3.7 nM. Anti-inflammatory was done employing the method described by Kirtikara et al.[12] Aspirin was used as reference and exhibited activity against COX-1 with IC_{50} value of 4–5 μg/mL and COX-2 with IC_{50} value of 9–10 μg/mL.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.05.070.

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