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New chlorinated peptides from the tropical marine sponge *Dysidea* sp.

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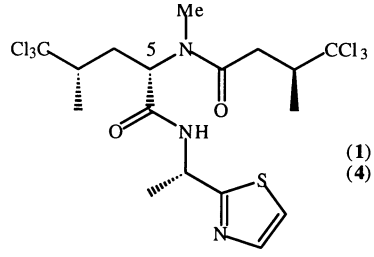
Abstract—Five new chlorinated peptides (**5**)– (**9**) have been isolated from a *Dysidea* sp. and identified by two-dimensional NMR spectroscopy. The absolute stereochemistry of the metabolites was deduced by chemical correlation with *S*-(−)-4,4,4-trichloro-3-methylbutanoic acid (**10**) and with an alcohol (**11**).

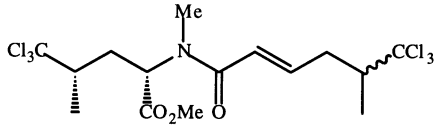
1. Introduction

The sponge commonly known as *Dysidea herbacea* occupies a distinctive place in the marine natural products literature since over 50 reports[1–5] document its diverse secondary metabolite profile. Over 20 of these papers address the structure and stereochemistries of chlorinated alkaloids; these metabolites are all characterised by the presence of a 5,5,5-trichloroleucine unit,[2–5] which can have either D or L stereochemistry. Examples of metabolites with 5*S* stereochemistry, i.e. the L-leucine series, include dysidenin **1**,[2a] herbaceamide **2**,[5a] and the *N*-methyldysideathiazole **3**;[5b] metabolites of the 5*R* series include isodysidenin **4**.[2b]

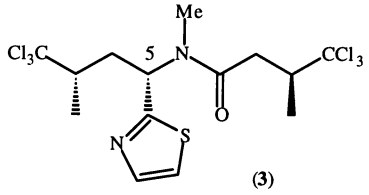
Collections of *Dysidea herbacea* made at Lizard Island recently yielded new sesquiterpene and brominated diphenyl ether metabolites.[1] These sponge samples have also yielded three new polychlorinated dipeptide methyl esters **5**–**7**, and their related acids **8**–**9**, which are the basis of the current report. Our results provide further insights into the effect of the C5 stereochemistry on the optical rotation of the metabolites.



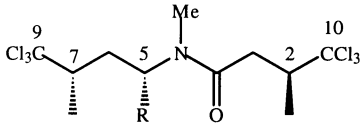
(**1**)
(**4**) epimer at C5



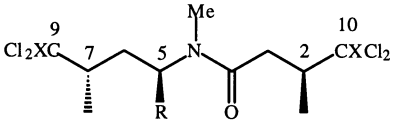
(**2**) R = Me



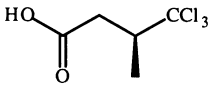
(**3**)



(**5**) R = CO_2Me
(**8**) R = CO_2H
(**11**) R = CH_2OH
(**13**) R = CO_2Et
(**15**) R = R = $CO_2{}^tBut$



(**6**) X = Cl; R = CO_2Me
(**7**) X = H; R = CO_2Me
(**9**) X = Cl; R = CO_2H
(**12**) X = Cl; R = CH_2OH
(**14**) X = Cl; R = CO_2Et
(**16**) X = Cl; R = $CO_2{}^tBut$



(**10**)

Keywords: chlorinated peptide; DIBAH; $NaBH_4$; NMR; sponges.
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Table 1. ^{1}H and ^{13}C NMR data for chlorinated dipeptide esters **5**–**7**

Atom no.	δ $^{13}C^{a}$ (**5**)	δ $^{1}H^{b}$ (**5**)	δ $^{13}C^{a}$ (**6**)	δ $^{1}H^{b}$ (**6**)	δ $^{13}C^{a}$ (**7**)	δ $^{1}H^{b}$ (**7**)
1	17.1	1.35 (3H, d 6.4)	17.3	1.38 (3H, d 6.5)	13.4	1.20 (3H, d 6.8)
2	51.5	3.28 (m 9.3, 6.4, 2.7)	51.5	3.29 (m 9.3, 6.4, 2.6)	40.1	2.80 (m 9.5, 6.8, 2.9)
3	37.2	3.10 (dd 16.0, 2.7)	37.4	3.03 (dd 16.0, 2.6)	37.7	2.65 (dd 16.5, 6.8)
		2.48 (dd 16.0, 9.3)		2.48 (dd 16.0, 9.3)		2.40 (dd 16.5, 9.5)
4	171.0	–	170.4	–	172.3	–
5	53.9	5.48 (dd 12.6, 3.6)	55.3	5.30 (dd 8.4, 5.6)	53.0	5.30 (m)
6	31.8	2.50 (m)	33.1	2.80 (m)	32.2	2.25 (ddd 14.5, 7.6, 6.1)
		2.02 (ddd 14.4, 10.7, 3.6)		1.50 (m)		1.66 (ddd 14.5, 9.7, 6.2)
7	52.0	2.20 (m)	52.2	2.80 (m)	41.4	2.11 (m)
8	16.0	1.37 (3H, d 6.4)	16.4	1.36 (3H, d 6.5)	12.6	1.18 (3H, d 6.6)
9	105.4	–	105.4	–	77.1	5.97 (d 2.7)
10	105.1	–	105.2	–	78.3	6.01 (d 2.9)
11	171.5	–	170.9	–	171.8	–
N-Me	31.5	2.98 (3H, s)	32.0	3.00 (3H, s)	31.8	2.93 (3H, s)
—*O*Me	52.6	3.72 (3H, s)	52.4	3.74 (3H, s)	52.2	3.71 (3H, s)

[a] 125 MHz; samples in $CDCl_3$ referenced to $CHCl_3$ at 77.0 ppm.
[b] 500 MHz; samples in $CDCl_3$ referenced to $CHCl_3$ at δ 7.25; data for major rotamer.

2. Results and discussion

Collections of *Dysidea* sp. 1524 from Horseshoe Reef yielded the chlorinated dipeptide methyl ester **5** and its acid **8**. A sample collected at the Sponge Bommie was extracted to give the chlorinated dipeptide methyl ester **6** and its acid **9**, while the didechloro ester **7** was isolated from a sample collected at the North Point dive site.

Identification of the chlorinated dipeptide ester **5** with an HRESMS of 447.9590 for $(M+H)^+$ corresponding to a molecular formula of $C_{13}H_{19}NO_3Cl_6$, was facilitated when its proton NMR data were compared with those of dysidenin **1**[2a] and isodysidenin **4**,[2b] which differ only in stereochemistry at H5 (Table 1). The presence of a —OMe signal (δ 3.72) in **5**, together with the lack of thiazole signals, suggested that the *N*-thiazolylethylamide moiety of **4** had been replaced by a methyl ester. An *N*-methyl signal was found at δ 2.98, while two doublets at δ 1.37 and 1.35 suggested the presence of methyl groups adjacent to CCl_3 groups. Signals at δ 3.28 (1H, ddd), 2.48 (1H, dd) and 3.10 (1H, dd) exactly matched those at the 2- and 3-positions of **1** and **4**, while signals at δ 5.48 (1H, ddd), 2.50 (1H, m), 2.20 (1H, m) and 2.02 (1H, m) corresponded closely to those at the 5–7-positions of **1**. ^{13}C NMR, DQFCOSY, and geHMBC data were fully in accordance with the proposed structure. In the dysidenin/isodysidenin series of metabolites, the chemical shifts of the H6 and H7 protons are sensitive to the stereochemistry at C5,[2b,2h,5b] while all the chlorinated alkaloids isolated to date have *S* stereochemistry at the trichloromethyl-substituted carbon centre.[5b,6] The C5 stereochemistry also influences the optical rotation; to date, all known metabolites of the dysidenin (5*S*) series have negative $[\alpha]_D$.[5b] A 2*S*, 5*S*, 7*S* configuration was provisionally assigned to **5** in view of its $[\alpha]_D$ and the strong similarity of its proton NMR data with those of dysidenin.

The second new dipeptide **6**, with HRESMS of 469.9405 for $M+Na^+$ corresponding to a molecular formula of $C_{13}H_{19}NO_3Cl_6$, gave closely similar NMR data to **5** (Table 1). The presence of an —OMe signal (δ 3.74) in **6**, together with the lack of thiazole signals, again suggested that the *N*-thiazolylethylamide moiety of **4** had been replaced by a methyl ester. An *N*-methyl signal was found at δ 3.00, while two doublets at δ 1.36 and 1.38 suggested the presence of methyl groups adjacent to CCl_3 groups. Signals at δ 3.29 (1H, ddd), 2.48 (1H, dd) and 3.03 (1H, dd) exactly matched those at the 2- and 3-positions of **1** and **4**, while signals at δ 5.30 (1H, ddd), 2.80 (2H, m) and 1.50 (1H, m) corresponded closely to those at the 5–7-positions of isodysidenin **4**.[2b] ^{13}C NMR, DQFCOSY, and geHMBC data were fully in accordance with the proposed structure. A 5*R* configuration was assigned to **6** in view of the strong similarity of its proton NMR data with those of isodysidenin. However, ester **6** had a small, negative optical rotation value, rather than the positive value anticipated for a metabolite of the isodysidenin series. We therefore sought confirmation of the configurations assigned to **5** and **6** by chemical means.

First, acid hydrolysis of **5** and **6** gave the anticipated (−)-4,4,4-trichloro-3-methylbutanoic acid **10**,[4b,4d,7] confirming *S* stereochemistry at C2. The C5 stereochemistry of the two esters **5** and **6** was investigated by sequential reduction of the ester group (DIBAH, −78°C, toluene; then $NaBH_4$, EtOH) to give diastereomeric alcohol products which were compared with the alcohol **11** prepared by Unson et al. during the course of an investigation into the stereochemistry of *N*-methyldysideathiazole (**3**). A 2*S*, 5*S*, 7*S* configuration for **11** had been confirmed by X-ray analysis of a *p*-bromophenyl carbamate derivative.[5b] The NMR data for the alcohol product derived from ester **5** matched those of **11**, while the alcohol **12** prepared from **6** was clearly different. In particular, the H5 signals were at δ 4.94 and 4.64 for the two alcohols derived, respectively, from **5** and **6**, compared to δ 4.92 for **11**. Thus esters **5** and **6** have 5*S* and 5*R* configuration, respectively.

The $[\alpha]_D$ for the alcohol sample from **5** was −32.5, which compared reasonably well with the literature value of −41 for **11**;[5b] however, the value for the epimeric alcohol **12** prepared from **6** was again negative (−26.2) instead of the positive value anticipated from literature trends. Hence the 5*R* series gives anomalous optical rotation data for both the methyl ester and alcohol. The ethyl ester analogues of **5** and **6** were available as side-products of the reaction

sequence. The conditions for ester reduction with DIBAL were designed to minimise epimerisation of the aldehyde products, and consequently led to incomplete reduction. Ethyl esters **13** and **14** then resulted from transesterification using $NaBH_4$/EtOH. The $[\alpha]_D$ values of the 5*S* and 5*R* ethyl esters were measured as −25.0 and +18.8, respectively. Optical rotation data for some synthetic chlorinated dipeptide *t*-butyl esters[2g] were also available. The 2*S*, 5*S*, 7*S* *t*-butyl ester **15** gave a negative $[\alpha]_D$ while the value for the 5*R* epimer **16** was positive.[8] Thus the $[\alpha]_D$ values of esters with bulky alkoxy groups fit the dysidenin/isodysidenin trend, whereas the smaller methoxy or hydroxymethyl substituents lead to an anomalous trend for the 5*R* series. The proton NMR data showed that the carboxylic derivatives **5**–**9** and the two alcohols **11** and **12** were all mixtures of rotamers about the amide or C—N bonds in $CDCl_3$ solution. The anomalous $[\alpha]_D$ values for **6**, **9** and **12** may reflect the conformational preferences available to this suite of compounds.

A third dipeptide ester **7**, with HRESMS of 402.0167 for $(M+Na)^+$ corresponding to $C_{13}H_{21}NO_3Cl_4Na$, was isolated. The dichloromethyl substituents suggested by this molecular formula were recognised by methine signals at 77.1 and 78.3 ppm for C9 and C10, and by the characteristic doublet signals at δ 5.97 and δ 6.01 for their attached protons.[2h] The remaining ^1H and ^{13}C signals (Table 1) were assigned by analysis of DQFCOSY and geHMBC spectra. Evidence for the proposed 5*R* stereochemistry was:

1. the ^1H NMR data closely match those for 9,11-didechloroisodysidenin;[2h]
2. the chemical shift and coupling constants of H5 correlate better with those of **6** than **5**.

A negative optical rotation (−22.2) was measured for **7**, consistent with the pattern of values for 5*R* methyl esters.

Peptides **5** and **6** were isolated together with the corresponding acids **8** and **9**, which are likely the genuine natural products, given the ease of transesterification detected during synthetic modifications. Thus the peptide esters **5**–**7** likely result from the use of methanol during the isolation sequence.[5d] The parent dipeptide acids are envisaged as the biosynthetic precursors leading to thiazole metabolites such as dysidenin or isodysidenin in this sponge–symbiont association.

3. Experimental

3.1. Isolation of metabolites

All sponge samples were collected using SCUBA at about 3–5 m depth at Horseshoe Reef, the Sponge Bommie, and at North Point, at Lizard Island on the Great Barrier Reef. A voucher sample of *Dysidea* sp. 1524 (G314231) is held at the Queensland Museum, Brisbane. A collection of this sponge (sample #21-2-99-2-1) from Horseshoe Reef, Lizard Island yielded the chlorinated dipeptide methyl ester **5** and its acid **8**; sponge sample 14-7-98-1-2 collected at the Sponge Bommie, Lizard Island was extracted to give the chlorinated dipeptide methyl ester **6** and its acid **9**, while sample 22-2-99-1-2 gave the didechloro ester **7**.

Frozen sponge (240 g wet wt; 21-2-99-2-1), collected at Horseshoe Reef, was cut into pieces and left in DCM/MeOH 1:1 (3×300 mL) at room temperature. The organic solution was filtered through a plug of cotton wool and the solvent removed by rotary evaporation to give a green semi-solid (1.2 g), which was further purified by flash chromatography on silica using a step gradient of hexane/DCM 1:1 to neat DCM, then DCM/EtOAc through neat EtOAc, then EtOAc/MeOH through neat MeOH. Early eluting fractions containing halogenated metabolites were further purified by silica HPLC using 5% EtOAc in hexane to give 38 mg of the chlorinated alkaloid **5** while later eluting fractions were purified by further flash chromatography using 40% EtOAc in hexane to yield 52 mg of dysidenin **1** and 45 mg of acid **8**. The Sponge Bommie collection (105 g wet wt; 14-7-98-1-2) was worked up in similar fashion to give 106 mg of the chlorinated alkaloid **6** and 18 mg of acid **9**.

3.1.1. Chlorinated peptide (5): methyl 5,5,5-trichloro-4-methyl-2-[methyl(4,4,4-trichloro-3-methyl-1-oxobutyl)-amino] pentanoate. Obtained as a white solid from diethyl ether/pentane, mp 66–68°C; $[\alpha]_D$=−16.1 ($CHCl_3$, c=0.02); for ^1H NMR and ^{13}C NMR, see Table 1; geHMBC, C1 with H2 and H3, C2 with H1 and H3, C3 with H1, C4 with *N*-Me and H3, C5 with *N*-Me and H6, C6 with H5, H7, and H8, C7 with H6 and H8, C8 with H5, H6, and H7, C9 with H6 and H7, C10 with H2 and H3, C11 with —*O*Me and H5; HRESMS, found 447.9590, $C_{13}H_{20}NO_3Cl_6$ $(M+H)^+$ requires 447.9569 (+4.7 ppm).

3.1.2. Chlorinated peptide (6): methyl 5,5,5-trichloro-4-methyl-2-[methyl(4,4,4-trichloro-3-methyl-1-oxobutyl)-amino] pentanoate. Obtained as a white solid from diethyl ether/pentane, mp 47–48°C; $[\alpha]_D$=−10.5 ($CHCl_3$, c=0.02); for ^1H NMR and ^{13}C NMR, see Table 1; geHMBC, C1 with H2 and H3, C2 with H1 and H3, C3 with H1, C4 with *N*-Me and H3, C5 with *N*-Me and H6, C6 with H5, H7, and H8, C7 with H6 and H8, C8 with H5, H6, and H7, C9 with H6 and H7, C10 with H2 and H3, C11 with —*O*Me and H5; HRESMS, found 469.9405, $C_{13}H_{19}NO_3Cl_6Na$ $(M+Na)^+$ requires 469.9388 (+3.5 ppm); GCMS *m/z* (intensity %) 388/390/392 (21/30/21), 330/332/334 (8/11/8), 260/262/264 (9/12/9), 202 (100), 166 (12), 123 (38), 102 (17) and 42 (58).

3.1.3. Chlorinated peptide (7): methyl 5,5-dichloro-4-methyl-2-[methyl(4,4,-dichloro-3-methyl-1-oxobutyl)-amino]pentanoate. Oil; $[\alpha]_D$=−22.2 ($CHCl_3$, c=0.0003); for ^1H NMR and ^{13}C NMR, see Table 1; geHMBC, C1 with H2 and H3, C2 with H1 and H3, C3 with H1, C4 with *N*-Me and H3, C5 with *N*-Me and H6, C6 with H5, H7, and H8, C7 with H6 and H8, C8 with H5, H6, and H7, C9 with H6 and H7, C10 with H2 and H3, C11 with —*O*Me and H5; HRESMS, found 402.0167, $C_{13}H_{21}NO_3Cl_4Na$ $(M+Na)^+$ requires 402.0168 (−0.2 ppm).

3.1.4. Chlorinated peptide (8): 5,5,5-trichloro-4-methyl-2-[methyl(4,4,4-trichloro-3-methyl-1-oxobutyl)amino] pentanoic acid. Obtained as white crystals from $CHCl_3$, mp 129–133°C; $[\alpha]_D$=−35.2 ($CHCl_3$, c=0.045); ^1H NMR

($CDCl_3$) δ 5.49 (1H, dd, J=12.4, 3.8 Hz, H-5), 3.28 (1H, ddd, J=9.4, 6.5, 2.6 Hz, H-2), 3.11 (1H, dd, J=16.1, 2.6 Hz, H-3), 3.02 (3H, s, *N*-Me), 2.55 (1H, m, H-6), 2.48 (1H, dd, J=16.1, 9.4 Hz, H-3), 2.25 (1H, m, H-7), 2.07 (1H, ddd, J= 14.7, 9.5, 5.6 Hz, H-6), 1.39 (3H, d, J=6.4 Hz, H-8), and 1.36 (3H, d, J=6.5 Hz, H-1); ^{13}C NMR ($CDCl_3$) 175.1 (C-11), 172.1 (C-4), 105.3 (C-9), 105.0 (C-10), 54.1 (C-5), 51.9 (C-7), 37.2 (C-3), 31.8 (C-6), 31.5 (*N*-Me), 17.1 (C-1) and 15.9 ppm. HRESMS, found 455.9234, $C_{12}H_{17}NO_3Cl_6Na$ $(M)^+$ requires 455.9232 (+0.4 ppm).

3.1.5. Chlorinated peptide (9): 5,5,5-trichloro-4-methyl-2-[methyl(4,4,4-trichloro-3-methyl-1-oxobutyl)amino] pentanoic acid. Obtained as white crystals from $CHCl_3$, mp 140–143°C; $[\alpha]_D$=−6.9 ($CHCl_3$, c=0035); ^{1}H NMR ($CDCl_3$) δ 5.23 (1H, dd, J=8.5, 5.6 Hz, H-5), 3.26 (1H, ddd, J=9.0, 6.3, 2.5 Hz, H-2), 3.04 (3H, s, *N*-Me), 3.03 (1H, dd, J=16.0, 2.1 Hz, H-3), 2.80 (2H, m, H-6 and H-7), 2.47 (1H, dd, J=16.0, 6.3 Hz, H-3), 1.57 (1H, ddd, J=14.7, 9.5, 5.6 Hz, H-6), 1.37 (3H, d, J=6.0 Hz, H-1), and 1.35 (3H, d, J=6.0 Hz, H-8); ^{13}C NMR ($CDCl_3$) 174.6 (C-11), 171.2 (C-4), 105.3 (C-9), 105.0 (C-10), 55.5 (C-5), 51.9 (C-7), 37.0 (C-3), 32.7 (C-6), 29.7 (*N*-Me), 17.2 (C-1) and 16.7 ppm. HRESMS, found 435.9380, $C_{12}H_{18}NO_3{}^{35}Cl_5{}^{37}Cl$ $(M+H)^+$ requires 433.9383 (−0.6 ppm).

3.2. Reduction of esters 5 and 6: 5*S* series

A solution of methyl 5,5,5-trichloro-4-methyl-2-[methyl-(4,4,4-trichloro-3-methyl-1-oxobutyl)amino] pentanoate **5** (10.0 mg) in dry toluene (3 mL) was treated with 1 M DIBAH in DCM (69 μL) at −78°C for 30 min, then the solution was allowed to warm to −40°C, quenched by addition of 1 M HCl (1 mL), and extracted into diethyl ether (3×3 mL). The organic layer was washed with 1 M HCl (3×3 mL), dried over Na_2SO_4, then evaporated to give an unstable aldehyde which was immediately dissolved in ethanol (1.5 mL) and reduced by treatment with a solution of 0.0058 M $NaBH_4$ in ethanol (120 μL). After stirring at room temperature for 45 min, the mixture was quenched by addition of acetone (1 mL). The solvent was removed, the residue dissolved in diethyl ether (2 mL) and washed with sat. aq. $NaHCO_3$ (2×1 mL), dried over Na_2SO_4, then evaporated to give alcohol **11** (2.4 mg, 26%) and ethyl ester **13** (0.5 mg, 5%) after separation by silica flash chromatography using 20% ethyl acetate in hexane.

3.2.1. Alcohol 11.[9] Oil; $[\alpha]_D$=−32.5 ($CHCl_3$, c=0.0012), lit. −40.8 ($CHCl_3$, c=1.78);[5b] ^{1}H NMR ($CDCl_3$) δ 4.94 (1H, m, H5), 3.78 (1H, dd, J=11.4, 4.8 Hz, H-11), 3.65 (1H, dd, J=12.9, 9.0 Hz, H-11), 3.28 (1H, m, H-2), 3.17 (1H, dd, J= 16.0, 2.0 Hz, H-3), 2.95 (3H, s, *N*-Me), 2.48 (1H, dd, J=16.5, 9.5 Hz, H-3), 2.28 (1H, m, H-7), 2.28 (1H, ddd, J=13.9, 8.0 and 2.0 Hz, H-6), 1.45 (1H, ddd, J=13.9, 10.5 and 3.2 Hz, H-6), 1.37 (3H,d, J=6.0 Hz, H-8) and 1.36 (3H, d, J=6.0 Hz, H-1). HREIMS, found 387.9352 $(M-CH_2OH)^+$, $C_{11}H_{16}NO_3{}^{35}Cl_6$ requires 387.9341 (−3.0 ppm).

3.2.2. Ethyl ester 13. Oil; $[\alpha]_D$=−25.0 ($CHCl_3$, c=0.005); ^{1}H NMR ($CDCl_3$) δ 5.47 (1H, dd, J=12.6, 3.6 Hz, H-5), 4.19 (2H, m, J=7.2 Hz, $-OCH_2CH_3$), 3.29 (1H, m, H-2), 3.10 (1H, dd, J=15.9, 2.7 Hz, H-3), 2.99 (3H, s, *N*-Me), 2.49 (1H, m, H-6), 2.23 (1H, m, H-7), 2.46 (1H, dd, J=15.9, 9.4 Hz, H-3), 2.03 (1H, ddd, J=14.3, 10.8, 3.6 Hz, H-6), 1.38 (3H, d, J=7.2 Hz, H-8), 1.36 (3H, d, J=6.4 Hz, H-1), and 1.27 (3H, t, J=7.2 Hz, $-OCH_2CH_3$); HRESMS, found 483.9554 $(M+Na)^+$, $C_{14}H_{21}NO_3Cl_6Na$ requires 483.9551 (−0.7 ppm).

3.3. Reduction of esters 5 and 6: 5*R* series

A solution of methyl 5,5,5-trichloro-4-methyl-2-[methyl-(4,4,4-trichloro-3-methyl-1-oxobutyl)amino] pentanoate **6** (11.0 mg) in dry toluene (3 mL) was treated similarly to give alcohol **12** (0.8 mg, 7%) and ethyl ester **14** (0.4 mg, 4%) after separation by silica flash chromatography using 20% ethyl acetate in hexane.

3.3.1. Alcohol 12. Oil; $[\alpha]_D$=−26.2 ($CHCl_3$, c=0.0008); ^{1}H NMR ($CDCl_3$) δ 4.64 (1H, m, H5), 3.78 (1H, dd, J=12.0, 4.0 Hz, H11), 3.65 (1H, dd, J=12.0, 9.0 Hz, H11), 3.29 (1H, m, H2), 3.03 (1H, dd, J=16.0, 2.0 Hz, H3), 2.98 (3H, s, *N*-Me), 2.57 (1H, m, H7), 2.46 (1H, dd, J=16.5, 9.5 Hz, H3), 2.28 (1H, ddd, J=13.5, 8.0 and 2.0 Hz, H6), 1.49 (1H, m, H6), 1.37 (3H,d, J=6.0 Hz, H1) and 1.36 (3H, d, J=6.0 Hz, H8); ^{13}C NMR ($CDCl_3$) 171.6 (C-4), 62.7 (C-11) 55.4 (C-5), 52.7 (C-7) 51.6 (C-2), 37.8 (C-3), 32.2 (C-6), 31.4 (−NMe), 17.3 (C-1), and 17.1 (C-8) ppm; signals for the $-CCl_3$ groups were not observed; HRESMS, found 419.9618 $(M+H)^+$, $C_{12}H_{20}NO_2Cl_6$ requires 419.9620 (−0.3 ppm).

3.3.2. Ethyl ester 14. Oil; $[\alpha]_D$=+18.5 ($CHCl_3$, c=0.0004); ^{1}H NMR ($CDCl_3$) δ 5.29 (1H, dd, J=8.5, 5.3 Hz, H-5), 4.20 (2H, m, J=7.2 Hz, $-OCH_2CH_3$), 3.30 (1H, m, H-2), 3.03 (1H, br d, J=15.8 Hz, H-3), 3.01 (3H, s, *N*-Me), 2.77 (2H, m, H-6, H-7), 2.48 (1H, dd, J=15.8, 9.6 Hz, H-3), 1.55 (1H, ddd, J=14.5, 9.5, 5.3 Hz, H-6), 1.38 (3H, d, J=6.8 Hz, H-1), 1.37 (3H, d, J=6.8 Hz, H-8), and 1.27 (3H, t, J=7.2 Hz, $-OCH_2CH_3$); HRESMS, found 483.9552 $(M+Na)^+$, $C_{14}H_{21}NO_3Cl_6Na$ requires 483.9550 (−0.3 ppm).

3.4. Hydrolysis of peptide 6

Ester **6** (5.1 mg) was refluxed with conc. HCl (0.5 mL) in glacial acetic acid (1 mL) for 20 h. After cooling to room temperature, water (2 mL) was added and the resulting solution extracted with CH_2Cl_2 (4×3 mL). The combined organic extracts were dried over $MgSO_4$, then concentrated to give 2.3 mg of acid **8**, which was further purified by flash chromatography on silica using 3% MeOH in $CHCl_3$ to give **8**, 1.1 mg 47%; white solid; $[\alpha]_D$=−16.1 ($CHCl_3$, c= 0.001), lit. −22 ($CHCl_3$, c=0.96).[5d]

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9. Revised proton assignments by DQFCOSY analysis.