Herbacic Acid, a Simple Prototype of 5,5,5-Trichloroleucine Metabolites from the Sponge *Dysidea herbacea*†

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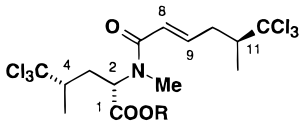
Dysidea herbacea, collected at Harrier Reef on the Great Barrier Reef, contains the novel metabolite herbacic acid as the major trichloroleucine metabolite. Herbacic acid appears to be an early product of direct free-radical chlorination of leucine and is a prototype for further transformation of the free carboxylic acid group and generation of complex trichloromethyl metabolites, including natural products of the dysidenin family.

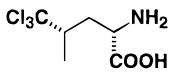
Some, but not all, specimens of the sponge *Dysidea herbacea* Keller 1889 (Dysideidae) collected in the Indo-Pacific contain unique 5,5,5-trichloroleucine derivatives, the structures of which vary with geographic location. These include dysidenin,[1] isodysidenin,[2] herbaceamide,[3] demethylisodysidenin,[4] related thiazoles,[5] and a variety of diketopiperazines (DKPs).[6,7] DKPs have also been isolated from *Dysidea chlorea* de Laubenfels 1954, collected near the island of Yap.[8] Recent interest in trichloromethyl-containing natural products was stimulated by biosynthetic observations that show that the pro-*S* methyl group of leucine, or an unidentified leucine derivative, is the origin of the trichloromethyl group in a natural product produced by the marine cyanobacterium *Lyngbya majuscula*.[9,10] This finding is important in the context that two independent groups[11,12] have provided evidence that another cyanobacterium, the symbiotic *Oscillatoria spongeliae*, which lives in association with *D. herbacea*, is the source of chlorinated metabolites extracted from *D. herbacea*. We now report the structure of (−)-herbacic acid (**1**)—including its absolute stereochemistry—from *D. herbacea* collected from Harrier Reef, Great Barrier Reef. Compound **1** occurs in large abundance (1.2% dry wt) in this sample of *D. herbacea*. Herbacic acid is also the first described trichloroleucine metabolite from *D. herbacea* with a free carboxyl group. As such, **1** may be a prototype intermediate that arises shortly after biohalogenation of the pro-4*S* methyl group of L-leucine that leads to the putative precursor (2*S*,4*S*)-5,5,5-trichloroleucine (**3**), but before the steps leading to more complex trichloromethyl natural products.

Samples of *D. herbacea* collected by hand (scuba) at Harrier Reef on the Great Barrier Reef, were immediately frozen and kept at −20 °C until needed. The lyophilized sponge was extracted exhaustively with methanol, and the MeOH extracts diluted with water before sequential partitioning against *n*-hexane, CCl_4, and $CHCl_3$. The *n*-hexane layer contained mostly fats, sesquiterpenes,[13] and herbaceamide (**2**) (0.11% dry wt).[3] Purification of the $CHCl_3$-soluble fraction by column chromatography (silica, MeOH−$CHCl_3$ gradient) afforded additional **2** and the major trichloroleucine metabolite, (−)-herbacic acid (**1**, 1.2% dry wt), which crystallized from hot acetonitrile as colorless prisms, mp 182−184 °C, $[\alpha]_D$ −36.3° (*c* 0.70, MeOH).

The DCIMS of **1** showed the expected isotope cluster for a hexachloro compound, and accurate mass measurement



1 R = H, *Herbacic acid*
2 R = Me, *Herbaceamide*



3

(*m*/*z* 459.9571 [M + H]$^+$) supported the formula $C_{14}H_{19}{}^{35}Cl_6NO_3$ for **1**. The ^{1}H NMR spectrum of **1** ($CDCl_3$) was virtually identical to that of **2**, with the exception of the absence of the signal due to the OMe group. The major differences between the ^{13}C NMR spectra of the two compounds were absence of the methoxy signal in **1** and change in the C-1 C=O signal from δ 171.1 ppm in **2** to 174.5 ppm in **1**. Finally, treatment of **1** with CH_2N_2 in diethyl ether−MeOH gave a nonpolar compound, identical in every respect to natural **2**. The ^{1}H and ^{13}C NMR assignments are analagous to those of **2** and were supported by a combination of gCOSY, gHMBC, and gradient-1D NOESY (GOESY[14]) experiments.

The 2*S*,4*S* configuration of **2** was known from earlier work;[3] however, the remote C-11 configuration was not determined. From the above correlation of **2** with **1**, it follows that both compounds must have the same configurations at both C-2 and C-4. Lemieux oxidation of **1** (aqueous $KMnO_4$, $NaIO_4$, aqueous MeOH) and silica chromatographic purification of the product gave the known (3*S*)-(−)-4,4,4-trichloro-3-methylbutanoic acid,[15] (*S*)-trichloroisovaleric acid (**5a**), establishing the 11*S* configuration for **1**. Thus, the configuration in **1** and **2** is 2*S*,4*S*,11*S*, which supports a conserved *S* configuration at the trichloroisopropyl group in those related chlorinated natural products determined independently.[5,16,17]

The structure of **1** represents the simplest derivative described, so far, of the hypothetical precursor 5,5,5-trichloroleucine (**3**) and is separated from **3** by acylation and methylation on nitrogen. Large amounts of **1** were detected in extracts of *D. herbacea* immediately after lyophilization and MeOH extraction. It is unlikely that **2** would hydrolyze to **1** during MeOH extraction; however, it is conceivable that small amounts of **2** may have arisen

† In memory of R. Bryan Miller, 1940−1998.

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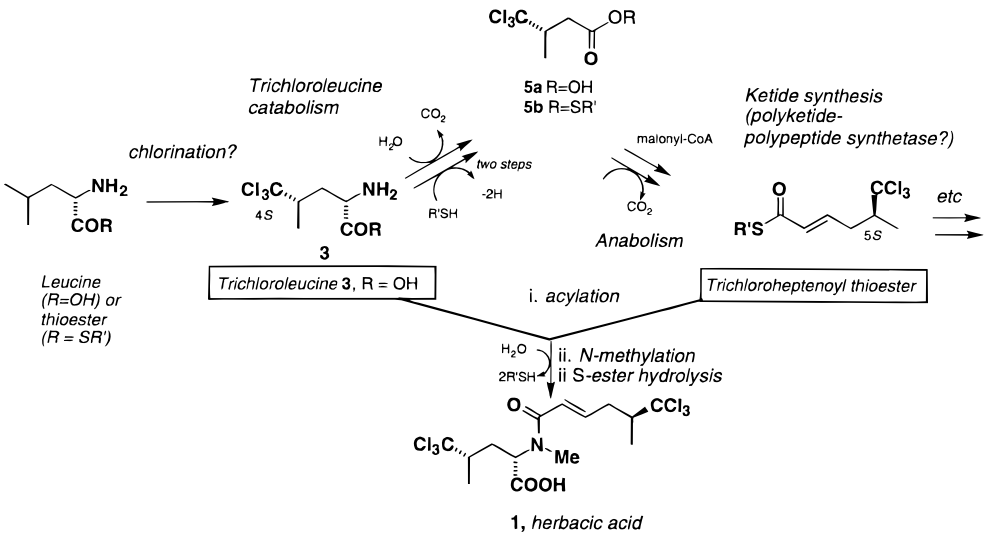
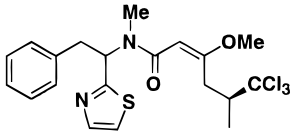
Scheme 1



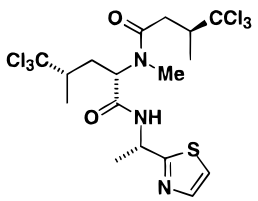
1, *herbacic acid*

by adventitious esterification of **1**. Given the great abundance of **1** (1.2% dry wt), it now appears more likely that **2** is an artifact of isolation and that **1** is the true natural product. It is interesting to note that, although we have detected known trichloromethyl metabolites in samples of *D. herbacea* collected from more than 10 locations, spanning 200 km of the Great Barrier Reef, we have only found **1** and **2** in the sponge at Harrier Reef.

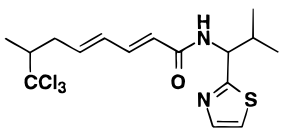
An important relationship between trichloroleucine metabolites of *D. herbacea* and those of free-living cyanobacteria is now evident. Administration of [1-^{14}C] leucine to *D. herbacea* resulted in incorporation of label into the trichloro-metabolite demethylisodysidenin, which supports leucine as its precursor.[18] Recent demonstration[9,10] that the trichloromethyl group of barbamide (**4**) from cultured *Lyngbya majuscula* derives from the pro-4*S* methyl group of (2*S*)-leucine adds support to an earlier hypothesis[3] that the trichloromethyl-containing *N*-acyl group in **2** is biosynthesized within the *Dysidea*−*Oscillatoria* assemblage from a degraded intermediate produced from **3** or an *N*-acyl derivative thereof (Scheme 1). The trichloro-2-isoheptenoate groups in **1** and **4** appear to arise from ketide chain extension of 4,4,4-trichloroisovaleroyl CoA (**5b**), a plausible catabolite of **3**.[3,9,10] The latter finds support in the fact that isovaleryl CoA is an early intermediate in the universal catabolism of leucine.[19] The close relationship of *N*-acyl amino acid **1** to the hypothetical intermediate **3** suggests that either (2*S*)-leucine, or more likely an *N*-acylated derivative thereof, is the primary biosynthetic substrate for introduction of chlorine at the unactivated C-4 position,[20] and that chlorination occurs early in the pathway from leucine to dysidenin and related metabolites found in the *Dysidea*−*Oscillatoria* association. It is interesting to note that other trichloro-substituted acyl groups are found in *D. herbacea* metabolites, including the trichloroisovaleroyl group (C_5) in dysidenin (**6**),[1] and the homologated trichloroisonona-2,4-dienoyl group (C_9) in herbamide (**7**).[21]



4 *Barbamide*



6 *Dysidenin*



7 *Herbamide*

Chlorine introduction may occur as early as free leucine, or a leucine thioester (Scheme 1), but if introduction of Cl occurs after *N*-acylation of leucine, the enzymic chlorination must exhibit a tolerance for variable *N*-acyl groups on leucine. Recent speculation on the biosynthesis of barbamide (**4**)[22] centers on the possibility of leucine chlorination via a "free-radical" pathway, involving a postulated enzyme with a novel mechanism. However, only partial details are known of the intervening steps from **3** to **4**,[23] and the enzyme responsible for chlorination of leucine has not been characterized. In light of the discovery of **1**, the former hypotheses can now be tested as it appears that easily prepared *N*-acyl (2*S*)-leucine derivatives should be suitable exogenous substrates for in vitro chlorination catalyzed by cell-free preparations of *Dysidea herbacea*−*Oscillatoria spongeliae* or *Lyngbya majuscula*.

Herbacic acid (**2**) did not exhibit antifungal activity against *Candida albicans* ATCC 4503, *C. glabrata*, *C. krusei*, or Fluconazole-resistant *C. albicans* 96-489 in microbroth dilution assays at concentrations <100 μg/mL.

Experimental Section

General Experimental Procedures. General procedures are described elsewhere.[24] Mass spectrometric measurements were performed at University of California, Riverside Mass Spectrometry facility. NMR measurements were carried out on either a Varian Mercury 300 or a Varian Inova 400 NB NMR spectrometer equipped with either a ^{1}H[^{15}N−^{31}P] pulsed-field gradient (PFG) indirect-detection probe or ^{1}H/^{13}C/^{15}N/^{31}P PFG auto-switchable probe. COSY, HMBC, TOCSY, and 1D

GOESY[14] experiments were carried out with gradient-enhanced pulse sequences.

Animal Material. *Dysidea herbacea* Keller, 1889 (Dysideidae) (sample: DEB-1) was collected from Harrier Reef (Great Barrier Reef, Australia, 15° 23′ S; 145° 45′ E, May 1990) by hand using scuba at −3 to −10 m, frozen immediately and stored for 2 months at −20 °C.

Collection and Extraction of *Dysidea herbacea*. The lyophilized tissue (68.7 g) was soaked in MeOH (800 mL) for 72 h and the solvent decanted. Fresh MeOH (600 mL) was added and the sponge allowed to stand for an additional 16 h. The MeOH extracts were combined, concentrated, and the H_2O content adjusted in steps from 10% (v/v), 20%, and 40% with sequential solvent partitioning at each step to obtain a *n*-hexane-soluble fraction (1.75 g), a CCl_4-soluble fraction (1.14 g), and a $CHCl_3$-soluble fraction (5.78 g). The *n*-hexane fraction was purified by silica chromatography to provide **2** (73.0 mg, 0.11% dry wt).[3]

A portion of the $CHCl_3$-soluble fraction (4.49 g) was applied to a Si gel column (5 × 25 cm) and eluted with a gradient of MeOH in $CHCl_3$. Small amounts of the sesquiterpenes 6-acetoxyfurodysin and 6-acetoxyfurodysinin[13] eluted first, followed by **2**.[3] Further elution of the column with 1:4 MeOH−$CHCl_3$ gave **1** (669 mg, 1.2% dry wt), a sample of which was crystallized from acetonitrile to give pure (−)-herbacic acid (**1**) as colorless prisms, mp 182−184 °C; $[\alpha]_D$ −36.3° (*c* 0.70, MeOH); UV (CH_3CN) λ_{max} 211 nm (ϵ 14 100); IR (film) ν_{max} 1731 cm^{-1}. In $CDCl_3$ solution **1** appears as a 14:1 mixture of rotamers about the amide bond. NMR data are given only for the major rotamer: ^1H NMR (300 MHz, $CDCl_3$) δ 6.98 (ddd, 1H, J = 14.9, 8.5, 6.3 Hz, H-9), 6.41 (dt, 1H, J = 15.0, 1.3 Hz, H-8), 5.50 (dd, 1H, J = 12.3, 3.7 Hz, H-2), 3.08 (s 3H, NCH_3), 3.07 (m, 1H, H-10a), 2.70 (m, 1H, H-11), 2.60 (dd, 1H, J = 13.1, 13.1 Hz, H-3a), 2.35 (m, 1H, H-10b), 2.31 (m, 1H, H-4), 2.09 (m, 1H, H-3b), 1.42 (d, 3H, J = 6.4 Hz, H-6), 1.36 (d, 3H, J = 6.6 Hz, H-13); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5 (s, C-1), 168.1 (s, C-7), 145.1 (d, C-9), 122.2 (d, C-8), 105.6 (s, C-5), 105.2 (s, C-12), 54.2 (d, C-11), 54.2 (d, C-2), 51.9 (d, C-4), 36.3 (t, C-10), 32.0 (q, NCH_3), 31.9 (t, C-3), 16.5 (q, C-6), 16.2 (q, C-13); DCI (NH_3) found m/z 459.9571 $[M + H]^+$, calcd for $C_{14}H_{20}{}^{35}Cl_6NO_3$, 459.9574.

Acid **1** was detected by TLC (R_f 0.27, 1:9 MeOH−$CHCl_3$, 0.02% bromocresol green spray) as the major component in a freshly prepared $CHCl_3$-soluble fraction of the sponge extract.

Methylation of (−)-Herbacic Acid−Herbaceamide (2). A solution of **1** (35 mg, 76 mmol) in MeOH (2 mL) was cooled to 0 °C in an ice-bath. Ethereal diazomethane was added until a permanent yellow color was obtained. After 10 min the solvent was removed under a stream of dry N_2 to give herbaceamide (**2**) (30.2 mg), $C_{15}H_{21}Cl_6NO_3$, identical with authentic **2** by ^1H NMR, MS, and $[\alpha]_D$ −34.6° (*c* 0.43, MeOH); lit.[3] −35° (*c* 0.55, MeOH).

Oxidation of (−)-Herbacic Acid: (−)-(3*S*)-4,4,4-Trichloro-3-methylbutanoic Acid (5a). (−)-(3*S*)-4,4,4-Trichloroisovaleric Acid. Aqueous solutions of potassium permanganate (2.0 mL, 0.11 M) and sodium periodate (1.5 mL, 0.12 M) were added to a solution of **1** (20.3 mg, 0.044 mmol) in 2:1 MeOH−H_2O (3 mL), and the mixture was stirred for 16 h at 25 °C. The mixture was treated with several drops of 6M aqueous, HCl concentrated, and extracted with EtOAc (3 × 25 mL). The combined EtOAc layers were dried (Na_2SO_4) and evaporated, and the residue was purified by chromatography, first on reversed-phase C_{18} silica (80:20 MeOH−H_2O), then over silica (3:97 MeOH−$CHCl_3$) to provide (*S*)-(−)-**5a** (1.2 mg), $[\alpha]_D$ −22° (*c* 0.96, $CHCl_3$); lit.[15] $[\alpha]_D$ −30° (*c* 0.4, $CHCl_3$), lit.[25] $[\alpha]_D$ −27.6° (*c*, 0.28, $CHCl_3$).

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