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Synthesis and X-ray Crystal Structure of the *Dolabella auricularia* Peptide Dolastatin 18[†,1]

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A previously synthesized unit of dolastatin 10 **(1)**, dolaphenine (Doe, **3**), was converted in four steps to tripeptide **10**. Subsequent condensation with carboxylic acid **11** (four steps from Meldrum's acid) provided a practical synthesis of the cancer cell growth inhibitor dolastatin 18 (**2**, Dhex-(*S*)-Leu-(*R*)-*N*-Me-Phe-Doe). The synthesis of dolastatin 18 **(2)** confirmed the *R* stereochemistry of the *N*-Me-Phe unit as originally assigned and unusual among amino acid components of the sea hare *Dolabella auricularia*. An X-ray crystal structure determination of dolastatin 18 was also completed.

The marine shell-less mollusk *Dolabella auricularia* contains a number of cancer cell line inhibitory and in vivo antineoplastic peptides that we began to isolate in 1972. One of these, the exceptionally active anticancer drug dolastatin 10 (**1**),[2] an unusual thiazole-containing pentapeptide that we isolated from a *D. auricularia* collection made in the Indian Ocean, is presently undergoing human cancer clinical trials under the auspices of the U.S. National Cancer Institute,[3] and very encouraging preclinical studies are also ongoing.[4] More recently, other research groups, especially that of Yamada,[2b,5] have considerably expanded the number and structural variety of *D. auricularia* bioactive constituents. In addition, it is now being realized that exogenous dietary sources employed by *D. auricularia,* such as cyanobacteria, might be responsible at least in part for the production of these most important dolastatin peptides and probably other classes.[6a,b] The isolation of symplostatins 1[6c] and 3,[6d] analogues of dolastatin 10, as well as peptide **1**[6c] itself from *Symploca* marine cyanobacteria and of dolastatin 16 from *Lyngbya majuscula*[6e] provides ample support for this viewpoint.

Results and Discussion

In 1983, we extended our investigation of *D. auricularia* anticancer constituents to specimens we collected in Papua New Guinea, which led to the isolation of the novel human cancer cell-growth-inhibitory dolastatins 16,[7a] 17,[7b] and 18.[7c] The composition and sequence assignment of dolastatin 18 was determined by means of carbon and proton NMR spectroscopy and analytical HPLC to be Dhex-L-Leu-*N*-Me-D-Phe-Doe (**2**), where Dhex (dolahexanoic acid) corresponds to the 2,2-dimethyl-3-oxohexanoyl unit and Doe (**3**, dolaphenine) refers to the C-terminal thiazole unit found in **1** and also in the cyanobacterial metabolite barbamide.[7d] Dolastatin 18 (**2**) was isolated in small quantity (1.51 mg), but a preliminary anticancer evaluation revealed significant inhibition of growth of several human cancer cell lines (e.g., NCI-H460, GI_{50} 0.39 μg/mL).[7c] Synthesis of **2** became necessary for confirmation of structure and further biological evaluations. A convergent pathway was devised whereby

† Dedicated to Professor Carl Djerassi on the occasion of his 80th birthday and in recognition of his world-class contributions to chemistry and medicine.

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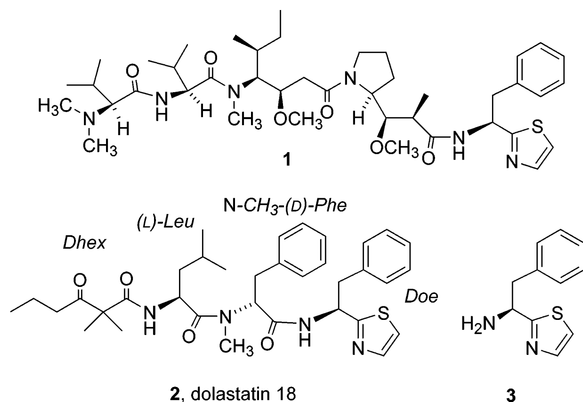
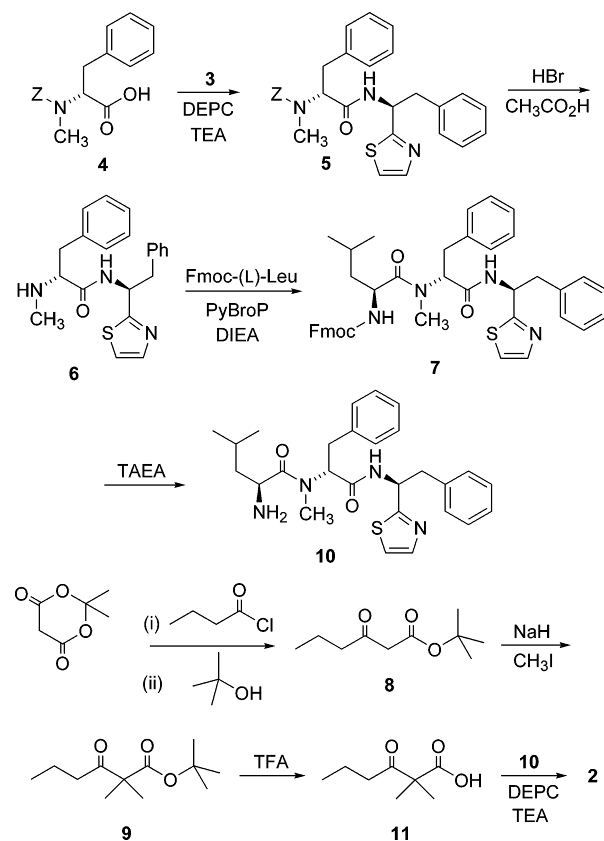


SCHEME 1



the C-terminal tripeptide and the dolahexanoic acid moieties could be coupled in the last step (Scheme 1). Treatment of *N*-benzyloxycarbonyl-(*R*)-phenylalanine with iodomethane and sodium hydride according to the method of Benoiton gave Z-*N*-Me-(*R*)-Phe (**4**) in good yield.[8] Synthesis of dolaphenine (**3**) was carried out according to earlier procedures.[2b,9] Condensation of amino acid **4** and amine **3** by use of diethyl phosphorocyanidate[10] (DEPC) in the presence of triethylamine gave the N-protected dipeptide **5**. Removal of the benzyloxycarbonyl group was performed by treatment with hydrogen bromide in acetic acid to give *N*-Me-(*R*)-Phe-Doe (**6**); the usual method for Z-cleavage using palladium over carbon with cyclohexene was precluded by the presence of the thiazole. An attempt to couple dipeptide **6** to Fmoc-(*S*)-Leu by treatment with BroP failed, but activation with PyBroP,[11] which has been shown to be successful in the coupling of hindered *N*-methylated amino acids, in the presence of diisopropylethylamine (DIEA) yielded Fmoc-(*S*)-Leu-*N*-Me-(*R*)-Phe-Doe (**7**). An X-ray analysis of peptide **7** confirmed its structure and stereochemistry.

Synthesis of *tert*-butyl 3-oxohexanoate (**8**) was accomplished cleanly according to the procedure of Yonemitsu and co-workers,[12] via reaction between Meldrum's acid and butyryl chloride followed by lysis of the product with *tert*-butyl alcohol. Methylation of ketone **8** with iodomethane and sodium hydride afforded *tert*-butyl dolahexanoate (**9**). Before the final coupling, the fluorenylmethoxycarbonyl group was removed from N-protected tripeptide **7** by treatment with trisaminoethylamine followed by isolation using a phosphate buffer[13] to yield amine **10**. The *tert*-butyl group was removed from ester **9** by treatment with trifluoroacetic acid[14] to yield carboxylic acid **11**. Amide formation between amine **10** and acid **11** by use of DEPC[10] in the presence of TEA gave Dhex-(*S*)-Leu-*N*-Me-(*R*)-Phe-Doe (**2**), whose identity with dolastatin 18 was demonstrated by TLC and NMR spectroscopy. A high-field (500 MHz) ^{1}H NMR spectrum of an equimolar mixture of the natural and synthetic specimens in $CDCl_3$ showed exact coincidence of the signals, except for an unidentified (impurity) singlet at δ 1.27 in the spectrum of the natural isolate.[15] Interestingly, an X-ray analysis of synthetic dolastatin 18 (**2**) showed that, in crystalline form, the peptide has a folded orientation like that of dolastatin 10 (**1**).[2b]

In the next stage of cancer cell-line evaluations, the activity of the synthetic compound was significant (P388: ED_{50} 7.11 μg/mL; human tumors: GI_{50} 2.7−4.1 μg/mL) but less than that of the natural isolate by a factor of nearly 10. Previously, we suggested that such a discrepancy in the activities of natural and synthetic marine invertebrate peptides may be due to an extremely active (such as dolastatin 16) but chemically undetected impurity that is present in the isolated material in a quantity indicated only by biological methods.[16] Further biological studies of dolastatin 18 (**2**) are in progress.

Experimental Section

***N*-Benzyloxycarbonyl-*N*-methyl-(*R*)-phenylalanine (4).** A solution of *N*-benzyloxycarbonyl-*(R)*-phenylalanine (2.67 g; 8.9 mmol) in THF (60 mL) was stirred under argon. Iodomethane (5.1 mL; 81.1 mmol) was added, and the mixture was cooled to 0 °C before the addition of sodium hydride (60%; 1.68 g from which the oil was removed by a hexane wash; 42 mmol).

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The suspension was stirred under argon at 0 °C to room temperature for 22 h. Ethyl acetate (50 mL) was then added, followed by water, dropwise, until the mixture became a clear solution. Removal of most of the solvent yielded a gummy emulsion, which remained following the addition of ether (30 mL) and water (100 mL). Citric acid (10%; 100 mL) was added to the slightly alkaline mixture, and the product was extracted into ethyl acetate (100 mL) with loss of the emulsion. The organic phase was washed successively with water (2 × 150 mL), sodium thiosulfate solution (5%; 100 mL), and water (50 mL) and was dried. Removal of solvent yielded a colorless gum (2.73 g; 97.5%): ^{1}H NMR (two conformers in a ratio of 2:1) δ 7.34−7.12 (m, 10 H), 5.11 and 5.03 (s, 2 H), 4.90 (m, 1 H), 3.35 (m, 1 H), 3.07 (m, 1 H), 2.86 and 2.79 (s, 3 H).

***N*-Benzyloxycarbonyl-*N*-methyl-(*R*)-Phe-Doe (5).** To a solution of Doe[9] (**3**; 0.117 g; 0.57 mmol) in DCM (5 mL) was added a solution of **4** (0.238 g; 0.76 mmol) in DCM (5 mL), and a further 5 mL of solvent was used for transfer. Triethylamine (0.25 mL; 1.77 mmol) was added, and the mixture was cooled to 0 °C before the addition of DEPC (1.0 mL; 6.13 mmol). The mixture was stirred for 20 min, at which time reaction was complete. After removal of solvent, the residue was fractionated by flash chromatography (8:1 hexane−acetone) to yield the product as a gum (0.280 g; 98%): ^{1}H NMR δ 7.72 (bs, 1 H), 7.33−7.10 (m, 17 H), 5.58 (dd, 1 H, J = 15 Hz, 7 Hz), 5.08 (brs, 2 H), 4.88 (m, 1 H), 3.26 (m, 3 H), 2.96 (m, 1 H), 2.78 (brs, 3 H); EIMS m/z (relative intensity) 499 (M^+), 296, 224, 91 (100); HRMS (FAB) m/z (exact mass) 500.2035 [$(MH)^+$, 100; calcd for $C_{29}H_{30}N_3O_3S$ 500.2008].

***N*-Methyl-(*R*)-Phe-Doe (6).** Amide **5** (0.270 g; 0.54 mmol) was treated with HBr in acetic acid (30%; 5 mL), and the mixture was stirred at ambient temperature for 1 h in a flask closed by a drying tube. Ether (35 mL) was added, causing immediate precipitation of the hydrobromide salt as a gummy solid, and the mixture was retained at 0 °C for 1.5 h. The mother liquor was decanted, and the gum was washed with ether (10 mL) that was then decanted. The salt was dissolved in satd sodium bicarbonate solution (20 mL), and the free amine was extracted with DCM (3 × 20 mL). The combined organic phase was washed with water (10 mL) and dried. Removal of solvent yielded a pale yellow oil (0.16 g; 0.43 mmol) that was used immediately in the next reaction: ^{1}H NMR δ 7.90 (d, 1 H, J = 8.1 Hz), 7.78 (d, 1 H, J = 3.3 Hz), 7.31−7.11 (m, 11 H), 5.65 (dt, 1 H, J = 8.4 Hz, 6.0 Hz), 3.43 (dd, 1 H, J = 13.8 Hz, 6.0 Hz), 3.25 (dd, 1 H, J = 13.8 Hz, 8.4 Hz), 3.16 (m, 2 H), 2.63 (m, 1 H), 2.01 (s, 3H).

***N*-(9-Fluorenylmethoxycarbonyl)-(*S*)-Leu-*N*-Methyl-(*R*)-Phe-Doe (7).** To a solution of dipeptide **6** (0.16 g; 0.43 mmol) in DCM (5 mL) were added PyBroP (301.9 mg; 0.65 mmol) and *N*-(9-fluorenylmethoxycarbonyl)-L-leucine (0.224 g; 0.63 mmol). The mixture was stirred under argon and cooled to 0 °C before the addition of DIEA (0.3 mL). Stirring was continued for 4 h. Removal of solvent yielded a yellow gummy solid (1.0215 g) that was dissolved in ethyl acetate (50 mL). The organic phase was washed successively with citric acid solution (10%; 20 mL), saturated sodium bicarbonate solution (20 mL), and water (20 mL) and dried. Removal of solvent yielded a pale yellow foam that was fractionated by flash chromatography (5:1 hexane−acetone) to yield peptide **7** as a colorless solid that crystallized from ethyl acetate (0.174 g; 58%): mp 164−165 °C; $[\alpha]_D$ +45.3 (c 0.38, $CHCl_3$); ^{1}H NMR (500 Mz, $CDCl_3$) δ 7.78 (bs, 1 H), 7.75 (d, 2 H, J = 7.0 Hz), 7.58−7.52 (m, 3 H), 7.40−7.37 (m, 2 H), 7.30−7.11 (m, 13 H), 5.65 (m, 1 H), 5.52 (dd, 1 H, J = 11.0 Hz, 5.0 Hz), 5.31 (d, 1 H, J = 8.0 Hz), 4.46 (m, 1 H), 4.40 (dd, 1 H, J = 10.5 Hz, 7.0 Hz), 4.27 (dd, 1 H, J = 10.5 Hz, 7.0 Hz), 4.17 (t, 1 H, J = 7.0 Hz), 3.47 (m, 1 H), 3.39 (dd, 1 H, J = 15.0 Hz, 5.0 Hz), 3.30 (m, 1 H), 2.92 (m, 1 H), 2.90 (s, 3 H), 1.22 (m, 1 H), 1.10 (m, 1 H), 0.85 (m, 1 H), 0.78 (d, 3 H, J = 6.0 Hz), 0.73 (d, 3 H, J = 6.0 Hz); HRMS (FAB) m/z (exact mass) 701.3145 [$(MH)^+$, 20; calcd for $C_{42}H_{45}N_4O_4S$ 701.3161]. Anal. Calcd for $C_{42}H_{44}N_4O_4S$: C, 71.97; H, 6.33; N, 7.99. Found: C, 71.37; H, 6.45; N, 7.83.

X-ray Crystal Structure Determination of *N*-Fmoc-(*S*)-Leu-*N*-Me-(*R*)-Phe-Doe (7). A thick, plate-shaped X-ray sample (~0.44 × 0.32 × 0.38 mm) grown from ethyl acetate was mounted on the tip of a glass fiber. Data collection was performed at 297 ± 1 K. Accurate cell dimensions were determined by least-squares fitting of 25 carefully centered reflections in the range of 35° < θ < 40° using Cu Kα radiation. Crystal data: $C_{42}H_{44}N_4O_4S$, fw = 700.87, orthorhombic, $P2_12_12_1$, a = 9.842(2) Å, b = 19.695(4) Å, c = 20.024(4) Å, V = 3881.6 (13) Å^3, Z = 4, ρ_c = 1.199 Mg/m^3, μ(CuKα) = 1.101 mm^{-1}, λ = 1.541 78 Å. All reflections corresponding to a complete octant ($0 \le h \le 11$, $0 \le k \le 23$, $0 \le l \le 23$) were collected over the range of $0 < 2\theta < 130°$ by use of the $\omega/2\theta$ scan technique. Friedel reflections were also collected (whenever possible) immediately after each reflection. Three intensity control reflections were also measured for every 60 min of X-ray exposure time and showed a max variation of −0.5% over the course of the collection. A total of 6995 reflections were collected. Subsequent statistical analysis of the complete reflection data set using the XPREP[17] program, verified that the space group was $P2_12_12_1$. After Lorentz and polarization corrections, merging of equivalent reflections and rejection of systematic absences, 6249 unique reflections (R_{int} = 0.0153) remained, of which 5871 were considered observed ($I_o > 2\sigma(I_o)$) and were used in the subsequent structure determination and refinement. Linear and anisotropic decay corrections were applied to the intensity data as well as an empirical absorption correction (based on a series of psi-scans).[18] Structure determination was readily accomplished with the direct-methods program SHELXS.[19] All non-hydrogen atom coordinates were located in a routine run using default values in that program. The remaining H atom coordinates were calcd at optimum positions. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement using SHELXL.[19] The H atoms were included, their U_{iso} thermal parameters fixed at 1.2 the U_{iso} of the atom to which they were attached and forced to ride that atom. The final residual R_1 value for **7** was 0.0527 for observed data and 0.0572 for all data. The goodness-of-fit on F^2 was 1.048. The corresponding Sheldrick R values were wR_2 of 0.1381 and 0.1430, respectively. A final difference Fourier map showed minimal residual electron density; the largest difference peak and hole being 0.437 and −0.233 e/Å^3, respectively. Final bond distances and angles were all within expected and acceptable limits. The absolute structure of peptide **7** could be assigned with certainty based upon the value of the Flack absolute structure parameter[20] (0.01, esd 2). Consequently, the chiral centers of peptide **7** can be assigned as follows: 18*S*,21*R*,24*S*.

***tert*-Butyl 2,2-Dimethyl-3-oxohexanoate (9).** To a stirred suspension of sodium hydride (60%; 0.23 g; 5.69 mmol) in DMF (10 mL under argon) at 0 °C was added, dropwise, a solution of *tert*-butyl 3-oxohexanoate[12] (**8**, 512.5 mg; 2.75 mmol) in DMF (3 mL). The mixture was stirred at 0 °C until evolution of gas ceased (10 min), and a solution of iodomethane (0.37 mL; 5.88 mmol) in DMF (3 mL) was added dropwise. The mixture was stirred for 18 h before being distributed between water (100 mL) and ether (100 mL). The ethereal layer was washed successively with sodium thiosulfate solution (10%; 40 mL) and water (50 mL) and dried. Removal of solvent yielded ketone **9** as a volatile colorless oil (215.2 mg; 36.5%): ^{1}H NMR δ 2.43

(17) XPREP−The automatic space group determination program in the SHELXTL (see ref 19).

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(t, 2 H, $J = 7.2$ Hz), 1.62 (m, 2 H), 1.45 (s, 9 H), 1.31 (s, 6 H), 0.91 (t, 3 H, $J = 7.5$ Hz); HRMS (APCI) m/z (exact mass) 213.1377 [(M − H)$^+$, 23; calcd for $C_{12}H_{21}O_3$ 213.1491].

2,2-Dimethyl-3-oxohexanoic Acid (11). To a solution of ketone **9** (103.6 mg; 0.48 mmol) in DCM (4 mL) that was stirred under argon and cooled to 0 ° C was added TFA (3 mL; 38.9 mmol). The mixture was stirred for 2.5 h and removal of solvent yielded the product as a pale yellow oil (70.1 mg; 0.44 mol) that was used without further purification in the final coupling reaction.

Dolastatin 18 (2, Dhex-L-Leu-*N*-Me-D-Phe-Doe). To a stirred solution of N-protected tripeptide **7** (0.116 g; 0.165 mmol) in DCM (5 mL) at ambient temperature under argon was added tris(2-aminoethyl)amine (1.3 mL; 8.34 mmol). The mixture was stirred for 1.5 h, during which time the yellow solution became cloudy. DCM (20 mL) was added, and the mixture was washed successively with saturated NaCl solution (2 × 10 mL) and phosphate buffer (10 mL; 5 mL). The aqueous layers were extracted with DCM (20 mL), and the combined organic solution (40 mL) was washed with water and dried. Removal of solvent yielded cream-colored solid **10** (92 mg) that was used without further purification as follows.

A solution of 2,2-dimethyl-3-oxohexanoic acid (**11**, 30 mg) in DCM (3 mL) was added to a stirred solution of tripeptide **10** (92 mg) in DCM (2 mL), and the mixture was cooled to 0 °C under argon. Triethylamine (1 mL) and DEPC (0.05 mL) were added, and the mixture was stirred for 2.5 h, warming to room temperature. Removal of solvent yielded a creamy oil that was fractionated by flash chromatography (5:1 hexane−acetone) to give dolastatin 18 (**2**, 40.5 mg; 0.065 mmol; 40% yield based on peptide **7**). Further purification on Sephadex LH-20 [hexane−toluene−methanol (3:1:1)] gave a colorless solid that crystallized from acetone−hexane in a cluster of needles: mp 108−109 °C; $[\alpha]_D$ + 44 (c 0.05 CH_2Cl_2); IR (neat) 3340.27, 1683.48, 1665.98, 1634.85, 1533.23 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$), δ 7.74 (d, 1 H, $J = 3.3$ Hz), 7.29−7.12 (m, 12 H), 6.25 (d, 1 H, $J = 6.0$ Hz), 5.60−5.56 (m, 1 H), 5.54 (dd, 1 H, $J = 11.5$, 5.5 Hz), 4.58−4.55 (m, 1 H), 3.42 (dd, 1 H, $J = 14.0$, 5.5 Hz), 3.38 (dd, 1 H, $J = 15.0$, 5.5 Hz), 3.24−3.20 (m, 1 H), 2.92 (s, 3 H), 2.94−2.90 (m, 1 H), 2.45−2.41 (m, 2 H), 1.56−1.51 (m, 2 H), 1.32 (s, 3 H), 1.30 (s, 3 H), 1.12−1.06 (m, 2 H), 0.86−0.83 (m, 1 H), 0.84 (t, 3 H, $J = 7.5$ Hz), 0.74 (d, 3 H, $J = 6.0$ Hz), 0.71 (d, 3 H, $J = 6.0$ Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 210.57, 174.06, 172.80, 171.62, 169.40, 142.71, 136.90, 136.71, 129.39, 128.75, 128.50, 128.48, 126.90, 126.72, 118.79, 57.13, 55.43, 52.85, 48.32, 41.12, 41.04, 40.04, 33.44, 31.02, 24.35, 22.95, 22.49, 22.42, 22.00, 17.08, 13.59; EIMS m/z (relative intensity) 618 (M$^+$, 27), 415 ([M − Doe]$^+$, 100); HRMS (FAB) m/z (exact mass) 619.3363 [(MH)$^+$, 100; calcd for $C_{35}H_{47}N_4O_4S$ 619.3318].

X-ray Crystal Structure Determination of Dolastatin 18 (2). A colorless, plate-shaped X-ray sample (~0.40 × 0.16 × 0.12 mm) grown from acetone−hexane was mounted on the tip of a glass fiber. Data collection was performed at 297 ± 1 K. Accurate cell dimensions were determined by least-squares fitting of 25 carefully centered reflections in the range of 35° < θ < 40° using Cu Kα radiation. Crystal data: $C_{35}H_{46}N_4O_4S$, fw = 618.82, monoclinic, $P2_1$, $a = 10.182(2)$ Å, $b = 19.978(4)$ Å, $c = 17.471(4)$ Å, $\beta = 99.47(3)°$, $V = 3505.6(12)$ Å^3, $Z = 4$, $\rho_c = 1.173$ Mg/m^3, μ(Cu Kα) = 1.147 mm^{-1}, $\lambda = 1.541\ 78$ Å, $F(000) = 1328$. All reflections corresponding to a complete octant ($0 \le h \le 11$, $0 \le k \le 23$, $20 \le l \le 20$) were collected over a range of $0 < 2\theta < 130°$, by use of the $\omega/2\theta$ scan technique. Friedel reflections were also collected (whenever possible) immediately after each reflection. Three intensity control reflections were also measured for every 60 min of X-ray exposure time and showed a max variation of −7.0% over the course of the collection. A total of 10615 reflections were collected. Subsequent statistical analysis of the complete reflection data set using the XPREP[17] program verified that the space group was $P2_1$. After Lorentz and polarization corrections, merging of equivalent reflections and rejection of systematic absences, 9708 unique reflections ($R_{int} = 0.0741$) remained, of which 6588 were considered observed ($I_o > 2\sigma(I_o)$) and were used in the subsequent structure determination and refinement. Linear and anisotropic decay corrections were applied to the intensity data as well as an empirical absorption correction (based on a series of psi-scans).[18] Structure determination was readily accomplished with the direct-methods program SHELXS.[19] All non-hydrogen atom coordinates were located in a routine run using default values in that program. The remaining H atom coordinates were calculated at optimum positions. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement using SHELXL.[19] The H atoms were included, their U_{iso} thermal parameters fixed at 1.2 the U_{iso} of the atom to which they were attached and forced to ride that atom. The final standard residual R_1 value for **2** was 0.1385 for observed data and 0.3437 for all data. The goodness-of-fit on F^2 was 1.425. The poor R value and goodness-of-fit observed for the structural model are presumably due to the apparent disorder exhibited by a number of atoms in the two independent peptide molecules present in the asymmetric cell unit. Thus, splitting of the atomic positions were suggested for the following atoms: C1 of one molecule and C1, C3, O5, C20, C24, C25, C42, and C44 of the second molecule. A final difference Fourier map showed some residual electron density, the largest difference peak and hole being 0.518 and −0.646 e/Å^3, respectively. However, the final bond distances and angles were all within expected and acceptable limits. The absolute structure of dolastatin 18 could be assigned with certainty based upon the value of the Flack absolute structure parameter[20] (−0.01, esd 6). Consequently, the chiral centers of dolastatin 18 can be assigned as follows: 12*S*,21*R*,32*S*.

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Supporting Information Available: X-ray crystallographic tables of atomic coordinates, bond lengths and angles, and anisotropic thermal parameters for peptide (**7**) and dolastatin 18 **(2)** and ^{1}H NMR spectra of the natural and synthetic dolastatin 18. This material is available free of charge via the Internet at http://pubs.acs.org.

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