Antineoplastic Agents. 278. Isolation and Structure of Axinastatins 2 and 3 from a Western Caroline Island Marine Sponge[1a]

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The Republic of Palau marine sponge *Axinella* sp. was found to be an exceptionally productive source of cell growth inhibitory substances. The strongly antineoplastic polyether macrocyclic lactones halichondrin B (1) and homohalichondrin B (2) were isolated in 1.2×10^{-6}% and 5.4×10^{-7}% yields, respectively. In addition to axinastatin 1 (3), two new and cytostatic (GI_{50} values of 0.35 to 0.0072 μg/mL against six human cancer cell lines) cycloheptapeptides designated axinastatins 2 (4) and 3 (5) were discovered in 1.4×10^{-6}% and 1.25×10^{-6}% yields. Structures were elucidated by high-resolution FABMS and tandem MS/MS techniques augmented by high-field (400 and 500 MHz) 2D-NMR spectral analyses. The absolute configurations were established by a combination of hydrolysis, derivatization, and chiral gas chromatographic methods.

In 1989 we described the first cell growth suppressing (P388 lymphocytic leukemia, PS cell line) peptide (hymenistatin 1, a cyclooctapeptide) from a marine sponge.[2a] Subsequently we summarized discovery of the PS cell line inhibitory cycloheptapeptides, axinastatin 1,[2b] stylostatin 1,[2c] and phakelliastatins 1[2d] and 2.[2e] Meanwhile marine sponges have been found to contain other bioactive peptides, e.g., a thrombin-slowing tetrapeptide[2f] and a protein phosphatase-inhibitory pentapeptide.[2g] Thus marine Porifera are becoming increasingly important sources of unusual peptides with significant biological activity.

Axinastatin 1 (3) was isolated from an *Axinella* sp. collected (first in 1979) in the Republic of Palau in the Western Caroline Islands. Extended bioassay (PS) directed separation of the more difficultly accessible cytotoxic components of this sponge has now resulted in the discovery of two new and cancer cell growth inhibitory cycloheptapeptides herein named axinastatins 2 (4) and 3 (5). A detailed account of our earlier independent discovery[2b] of the exceptionally potent polyether antineoplastic agents halichondrin B (1) and homohalichondrin B (2), first reported and named by Uemura,[3] and the more recent isolation and structural determinations of axinastatins 2 and 3 follow.

Because of initial difficulties experienced in isolating enough of the *Axinella* sp. antineoplastic constituents for extended biological studies, it became necessary by 1985 to recollect 480 kg (wet wt) of this very productive source of antineoplastic substances. The present contribution is based on utilization of this recollection. The 2-propanol preserving solvent was removed and replaced with 1:1 dichloromethane–2-propanol. After some 3 weeks, the extract was collected and fresh solvent was added for another extraction cycle. The original aqueous–2-propanol extract was extracted with dichloromethane, and this fraction was combined with chlorocarbon fractions from the two additional extractions and subjected to solvent partition separation processes guided by bioassay (PS) as outlined in the Experimental Section.

Comprehensive gel permeation and partition column chromatographic procedures on Sephadex LH-20 followed by HPLC separations utilizing by bioassays established that the most potent constituents in this sponge were halichondrin B (1) and homohalichondrin B (2) present in very low concentrations.[2b] In addition, new cyclic peptides, axinastatins 2 and 3, were uncovered. Previously, 0.9 mg of halichondrin B and 0.4 mg of homohalichondrin B were obtained, and the structure of axinastatin 1 (3) was determined. We have now completed a scaleup isolation of halichondrin B and homohalichondrin B as well as the isolation and structure determination of axinastatins 2 and 3. These isolations were challenging because the latter peptides differed by only one methylene group, occurred in trace amount, and required a relatively complex series of procedures (summarized in the supplementary material Scheme 1, Parts 1–5).

Due to the trace concentrations of the halichondrins, they were not easily detected by TLC until their fractions became fairly concentrated. However, it was found that even low-concentration halichondrin fractions could be recognized by employing ^1H NMR. The exocyclic methylene signals in the ^1H NMR spectra at δ 4.7–5.2 ppm proved to be quite characteristic for halichondrin-type compounds. Also characteristic were the blue-black spots obtained on TLC plates upon spraying with ceric sulfate and heating. The peptides which helped complicate separation of the halichondrins appeared as yellow-orange spots. By combining the ^1H NMR and TLC detection methods, it became practical to concentrate the halichondrins by various column chromatographic techniques. Final separation was achieved by an HPLC system carefully developed for separation of halichondrin B and homohalichondrin B employing a semipreparative reversed-phase silica gel column with 5:5:6 acetonitrile–methanol–water as eluant (refractive index detector). These procedures led to the isolation of additional halichondrin B (5.8 mg, 1.2×10^{-6}% yield) needed for clinical development, and 2.6 mg (5.4×10^{-7}% yield) of homohalichondrin B. Both halichondrin B (1) and homohalichondrin B (2) gave high-field NMR (400 MHz) data consistent with those recorded by Uemura.[3] Furthermore halichondrin B (1) was found to be identical with a specimen provided by Prof. Uemura.[3]

Axinastatin 2 exhibited strong *in vitro* cytotoxicity against the murine leukemia P388 cell line (ED_{50} 0.02 μg/

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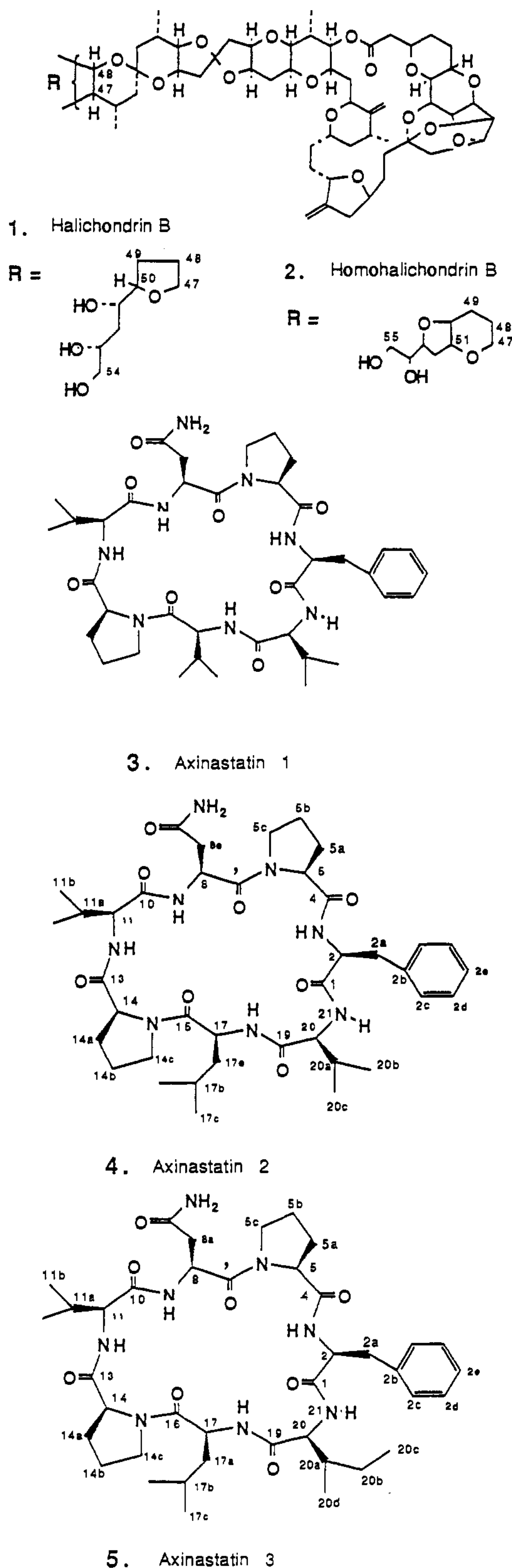
1. Halichondrin B

2. Homohalichondrin B

3. Axinastatin 1

4. Axinastatin 2

5. Axinastatin 3

mL) and a series of human cancer cell lines (Table 1). Accordingly, the structural elucidation of axinastatin 2 (4), mp 280–2 °C, $[\alpha]_D$ −153°, became an important objective. The high-resolution FAB molecular ion suggested the molecular formula $C_{39}H_{58}N_8O_8$. Interpretation (see below as illustrated for axinastatin 3) of the high-field (400-MHz) 2D-NMR spectra (COSY and ^{13}C–^1H correlation) indicated the presence of single asparagine, phenylalanine, and leucine units and two units each of proline and valine (see supplementary material Table 2 for details). Amino acid analyses of axinastatin 2 also pointed to Asp, Phe, Leu, Pro, and Val in a ratio of 1:1:1:2:2. The cycloheptapeptide amino acid sequence of axinastatin 2 was mainly determined by MS/MS[4] analyses which established the sequence as: cyclo(-Pro-Val-Asn-Pro-Phe-Val-Leu-). Protonation of the two proline and asparagine units by FAB mass spectrometry produced three series of fragmentations interpreted by MS/MS methods:[4]

Table 1. Cytostatic Activity of Axinastatins 2 and 3 Against Selected Human Tumor Cell Lines

		GI-50 (μg/mL)	
cell type	cell line	axinastatin 2 (4)	axinastatin 3 (5)
ovarian	OVCAR-3	0.058	0.0072
CNS	SF-295	0.35	0.18
renal	A498	0.38	0.11
lung-NSC	NCI-H460	0.19	0.033
colon	KM20L2	0.23	0.055
melanoma	SK-MEL-5	0.068	0.012

[a] GI = growth inhibition.

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m/z:  197   311   408   555   654
Pro - Val - Asn - Pro - Phe - Val - Leu

     m/z:         212   359   458   571   668
            Asn - Pro - Phe - Val - Leu - Pro - Val

            m/z:        245   344   457   554   653
                  Pro - Phe - Val - Leu - Pro - Val - Asn
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Since they were derived from ring opening at three sites, these fragments also helped establish axinastatin 2 as a cyclic peptide. The cyclic structure of axinastatin 2 was also supported by interpretation of unsaturation numbers in conjunction with mass spectral and NMR data. Eight carbonyl groups, one phenyl ring, and two proline rings account for only 14 unsaturation numbers. Hence the remaining one could only be attributed to a cyclic peptide ring.

The chirality of the amino acid units and absolute configuration of axinastatin 2 was established as follows. Axinastatin 2 was hydrolyzed by the propionic acid–hydrochloric acid method,[5] and the released amino acids were transformed into *N*-[(pentafluoropropyl)isopropyl] ester derivatives and analyzed by chiral GC.[6] All of the amino acids proved to represent the (*S*)-configuration, and structure 4 was assigned to axinastatin 2.

Axinastatin 3 also exhibited significant activity (ED_{50} 0.4 μg/mL) against the PS leukemia cell line and showed a higher level of activity (Table 1) than axinastatin 2 against human ovarian, lung, and colon cell lines. Consequently the structural elucidation of this peptide became important. Axinastatin 3 (5), recrystallized as needles from methanol–water: mp 291–4 °C; $[\alpha]_D$ −185°. The high-resolution fast atom bombardment mass spectrum analysis suggested the molecular formula $C_{40}H_{60}N_8O_8$. The presence of seven amino acid units was confirmed by detailed interpretation of the 400- and 500-MHz 2D-NMR spectra which included COSY, ^{13}C–^1H correlation, and HMBC. For example, the $^1H/^{13}C$ signals at δ 4.34/57.90, 3.99/64.73, 4.60/51.60, 3.78/64.41, 4.29/62.58, 4.30/53.46, and 4.21/57.90 were attributed to the α-proton and carbon signals of seven amino acids (supplementary material Table 3). The two exchangeable proton signals at δ 6.71, 6.07 (both broad singlets) and the signals at δ 3.02 (2H)/37.24 were assigned to an asparagine unit. The presence of a phenyl-

alanine unit was evident by the monosubstituted ^{13}C phenyl signals at δ 140.40, 131.00, 130.06, and 128.26, the ^{1}H signals at δ 7.26 and 7.19, and the $^{13}C/^{1}H$ signals at δ 39.18/3.10, 2.99. The presence of two proline units was revealed by two sets of spin systems: predominantly the signals at δ 3.65, 3.44 and 3.49, 3.36, respectively. While three of the six methyl ^{1}H NMR signals were overlapped, they were recognized using the ^{13}C NMR data as belonging to two methyl groups each of valine, leucine, and isoleucine. Results of axinastatin 3 amino acid analyses (*cf.* 4 above) confirmed the presence of Asn, Val, Phe, Ile, Leu, and Pro in a ratio of 1:1:1:1:1:2.

A tandem mass spectral study of axinastatin 3 established the amino acid sequence as cyclo(-Pro-Val-Asn-Pro-Phe-Ile-Leu-). The mass spectral fragments pointing to this sequence, together with an additional two sets of mass fragments, supported a cyclic rather than a linear peptide structure. A summary of the key mass fragmentation now follows:

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m/z   197   311   408   555   668

Pro - Val - Asn - Pro - Phe - Ile - Leu

      m/z         212   359   472   585   682

            Asn - Pro - Phe - Ile - Leu - Pro - Val

            m/z         245   358   471   568   667

                  Pro - Phe - Ile - Leu - Pro - Val - Asn
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The amino acid sequence was also supported by deductions from the HMBC and NOESY NMR spectra. That is, the H-20 (δ 4.21) and H-21 (δ 7.34) signals were found to be correlated with a carbon signal at δ 173.20 assigned to C-1. Other important correlations proved to be as follows: H-18 at δ 7.18 with C-19 at δ 173.84; H-3 at δ 7.91 with C-4 at δ 172.74; H-9 at δ 7.92 with C-7 and C-10 at δ 171.46 and 172.88; and H-12 at δ 7.92 with C-13 at δ 172.74. Due to the lack of exchangeable protons in the proline units, a NOESY spectrum was helpful in connecting one of the two proline groups to Asn: the signal at δ 4.60 showed cross peaks with the signal at δ 3.65 (H-5c), indicating the connectivity of N-6 to C-7 (supplementary material Table 4). Thus, the only position possible for the other proline was bridging between Val and Leu portions.

The structures determined for axinastatins 1–3 (**3–5**) provide a very helpful view of structure/activity relationships among the first three members of this new series of cell growth inhibitory cyclic peptides. Clearly, the seemingly minor substitutions of Leu and Ile for Val significantly increase the cell growth inhibitory properties. What relationship (such as serving as a carrier for a halichondrin in a trace quantity too small to detect spectrally), if any, these cytotoxic cycloheptapeptides have to the exceptionally potent antineoplastic halichondrins and what roles they play in the sponge represent interesting questions for the future. Whether they are endogenous biosynthetic products of the sponge or its symbionts or arise from exogenous food sources poses another interesting avenue of investigation. Presently we are further evaluating the antineoplastic potential of peptides **3–5**.

Experimental Section

General Experimental Procedures. All chromatographic solvents were redistilled. Sephadex LH-20 was from Pharmacia Fine Chemicals. Commercial TLC silica gel plates were obtained from Analtech, Inc. For HPLC separations, a Phenomenex Prepex C-8 reversed-phase column (Θ 10.0 mm × 25 cm) was used with a refractive index detector and acetonitrile–methanol–water (5:5:6) as eluant. Melting points were recorded with a Kofler-type melting point apparatus and were uncorrected. All NMR spectra were recorded employing a Bruker AM-400 spectrometer except for the HMBC spectra which were obtained with a Varian VXR-500S instrument.

***Axinella* sp. Collection and Preliminary Experiments.** Approximately 480 kg (wet wt) of the marine sponge *Axinella* sp. (order Axinellida, class Demospongiae)[2b] was recollected in March 1985 in the Republic of Palau (Western Caroline Islands). Voucher specimens are maintained in the Arizona State University Cancer Research Institute, the Northern Territory Museum (Darwin, Australia), and the Zoology Department, Smithsonian Institution (Washington, D.C.). The sponge was found as an irregular mass, brownish-yellow in color, and was preserved in 80% 2-propanol. After 11 months at ambient temperature, the shipping solution was drained and the 2-propanol was evaporated under reduced pressure. The 30 L of aqueous concentrate was partitioned with 15 L (8×) of dichloromethane to give the first active extract. The sponge was extracted with 640 L of dichloromethane–2-propanol (1:1), and this process was repeated to obtain the second and the third extracts. These extracts were reduced under vacuum to an aqueous concentrate, which was partitioned three times with dichloromethane. The water-soluble fraction was found to be inactive against the P388 cell line and was not pursued. The three dichloromethane fractions were combined, dissolved in methanol–water 9:1), and extracted with hexane. The hexane extract was not P388 cell line active (ED_{50} >100 μg/mL). The methanol–water solution was diluted to 3:2 and was extracted with dichloromethane to yield an active fraction (PS ED_{50} 0.29 μg/mL). The methanol–water layer was only marginally active (ED_{50} 19 μg/mL).

Isolation of Halichondrin B (1) and Homohalichondrin B (2). The active dichloromethane fraction was chromatographed on a Sephadex LH-20 column packed in dichloromethane–methanol (1:1), and the column was eluted with the same solvent to yield six fractions (supplementary material Scheme 1, Part 2, A1–A6). The active compounds were concentrated in the third (A3, PS ED_{50} 0.30 μg/mL) and fourth (A4, PS ED_{50} 0.44 μg/mL) fractions. The third fraction (A3) was subjected to separation on a Sephadex LH-20 column packed in methanol resulting in eight fractions. The third, fourth, and fifth fractions were PS cell line active (ED_{50} 0.58, 0.16, and 0.24 μg/mL, respectively). The three active fractions were combined, chromatographed on a column of Sephadex LH-20, and eluted with hexane–toluene–methanol (3:1:1). Eight fractions were recognized from this column. The third (C3, PS ED_{50} 0.021 μg/mL) and fourth fractions (C4, PS ED_{50} 0.28 μg/mL) were further separated.

Fraction C3 was separated by medium pressure (LoBar) chromatography on a RP-8 size B column using an acetonitrile–water linear gradient from 1:3 to 3:1, resulting in six fractions. The third fraction was further separated on a column of Sephadex LH-20 in dichloromethane–methanol (95:5) to afford five fractions. Final separation of halichondrin B and homohalichondrin B was achieved by HPLC using a reversed-phase (RP-8) silica gel column and acetonitrile–methanol–water (5:5:6) as eluant; halichondrin B (5.8 mg, 1.2×10^{-6}% yield) and homohalichondrin B (2.6 mg, 5.4×10^{-7}% yield) were obtained.[2b]

Isolation of Axinastatins 2 and 3 (4 and 5). The fourth fraction (C4) contained a relatively large proportion of peptides. Separation of fraction C4 (supplementary material Scheme 1, Part 4) on a LoBar size B RP-8 column using the acetonitrile–water linear gradient (1:3 → 3:1) gave six fractions (F1–F6). The second fraction was the most active (PS ED_{50} 0.03 μg/mL) and contained a concentration of peptides. One component of this fraction crystallized from dichloromethane yielding axinastatin 1 (**3**, 100 mg).[2b]

The mother liquor from the crystallization was chromatographed on a Sephadex LH-20 column using dichloromethane–methanol (95:5). Crystallization was used to remove another 33 mg of axinastatin 1 from an active fraction. Column chromatography of the residue on Sephadex LH-20 using cyclohexane–2-propanol–methanol (8:1:1) led to another concentration of PS cell line activity. HPLC separation of the active fractions from this column as summarized in Scheme 1, Part 5 (supplementary

material) afforded axinastatin 2 (5.3 mg) and additional axinastatin 1 (14 mg, or 147 mg total, 3.1×10^{-5}%). Repeated separation of fractions containing the complex mixtures of peptides using Sephadex LH-20 column chromatography, HPLC, and crystallization as noted in supplementary material Scheme 1, Part 5 finally yielded axinastatin 3 (6.0 mg, 1.25×10^{-6}%) and axinastatin 2 (6.7 mg, 1.4×10^{-6}% total yield).

Characterization of Axinastatin 2. The cyclic peptide was obtained as colorless crystals from methanol–water: PS ED_{50} 0.02 μg/mL (6.7 mg); mp = 280–2 °C; $[\alpha]_D = -153°$ (*c* 0.17, CH_3OH); HRFABMS calcd for $C_{39}H_{59}N_8O_8$ 767.4456 $[M + H]^+$, found 767.4464 (deviation 1.05 ppm); FABMS/MS *m/z* 767 $[[M + H]^+$, 654 $[M + H - Leu]^+$, 555 $[M + H - Leu - Val]^+$, 408 $[M + H - Leu - Val - Phe]^+$, 311 $[M + H - Leu - Val - Phe - Pro]^+$, 197 $[M + H - Leu - Val - Phe - Pro - Asn]^+$, 668 $[M + H - Val]^+$, 571 $[M + H - Val - Pro]^+$, 458 $[M + H - Val - Pro - Leu]^+$, 359 $[M + H - Val - Pro - Leu - Val]^+$, 212 $[M + H - Val - Pro - Leu - Val - Phe]^+$, 653 $[M + H - Asn]^+$, 554 $[M + H - Asn - Val]^+$, 457 $[M + H - Asn - Val - Pro]^+$, 344 $[M + H - Asn - Val - Pro - Leu]^+$, 245 $[M + H - Asn - Val - Pro - Leu - Val]^+$; and IR (NaCl, ν_{max}) 3316, 2961, 2874, 1645 (strong, broad), 1539, 1435 cm^{-1}.

Approximately 0.2 mg of axinastatin 2 was hydrolyzed with 1:1 propionic acid–hydrochloric acid at 140 °C for 30 min. The residue was transformed into 2-propyl ester derivatives using 2-propanol–acetyl chloride at 110 °C for 1 h. The 2-propyl esters were allowed to react with pentafluoropropionic anhydride to yield the *N*-(pentafluoropropionyl) derivatives which were injected into a gas chromatographic column composed of Chirasil-Val III (50 m × 0.25 mm, fused silica open tube capillary column). The sample was compared directly and indirectly with chiral amino acid standards. That procedure established the presence of all (*S*)-amino acids, namely, L-Val, L-Leu, L-Pro, L-Phe, and L-Asp in the hydrolysate.

Characterization of Axinastatin 3. The colorless specimen (6.0 mg) that crystallized as needles from methanol–water exhibited PS ED_{50} 0.4 μg/mL: mp 291–4 °C; $[\alpha]_D$ −185° (*c* 0.21, CH_3OH); HRFABMS *m/z* calcd for $C_{40}H_{61}N_8O_8$ 781.4612 $[M + H]^+$, found 781.4620; FABMS/MS *m/z* 781 $[M + H]^+$, 668 $[M + H - Leu]^+$, 555 $[M + H - Leu - Ile]^+$, 408 $[M + H - Leu - Ile - Phe]^+$, 311 $[M + H - Leu - Ile - Phe - Pro]^+$, 197 $[M + H - Leu - Ile - Phe - Pro - Asn]^+$, 667 $[M + H - Asn]^+$, 568 $[M + H - Asn - Val]^+$, 471 $[M + H - Asn - Val - Pro]^+$, 358 $[M + H - Asn - Val - Pro - Leu]^+$, 245 $[M + H - Asn - Val - Pro - Leu - Ile]^+$, 682 $[M + H - Val]^+$, 585 $[M + H - Val - Pro]^+$, 472 $[M + H - Val - Pro - Leu]^+$, 359 $[M + H - Val - Pro - Leu - Ile]^+$, 212 $[M + H - Val - Pro - Leu - Ile - Phe]^+$; and IR (NaCl, ν_{max}) 3584, 3325, 2960, 2893, 1645, 1523, 1437 cm^{-1}.

A 0.1-mg sample of axinastatin 3 was subjected to acid hydrolysis and formation of the 2-propyl ester *N*-(pentafluropropionyl) derivatives as summarized above for axinastatin 2. Subsequent chiral GC analysis (see above) resulted in (*S*)-amino acid assignments for axinastatin 3.

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Supplementary Material Available: Separation Scheme 1, Parts 1–5 and Tables 2–4 summarizing high-field 2D-NMR assignments (9 pages). Ordering information is given on any current masthead page.

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