The Isolation and Structure Elucidation of Tasiamide B, a 4-Amino-3-hydroxy-5-phenylpentanoic Acid Containing Peptide from the Marine Cyanobacterium *Symploca* sp.

Philip G. Williams,[†] Wesley Y. Yoshida,[†] Richard E. Moore,*[,†] and Valerie J. Paul[‡,§]

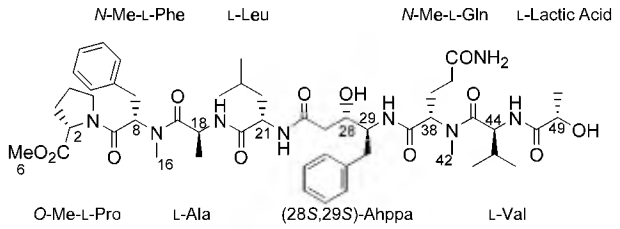
Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822, and University of Guam Marine Laboratory, UOG Station, Mangilao, Guam 96913

Received March 14, 2003

A new cytotoxic peptide, which displayed an IC_{50} value of 0.8 μM against KB cells, has been isolated from the marine cyanobacterium *Symploca* sp. The planar structure of tasiamide B (**1**), deduced by 2D NMR experiments, contains the unusual amino acid-derived residue 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa). The configuration of **1** was deduced by HPLC analysis of degradation products.

Serious investigations into natural products from marine organisms started in the 1960s. Now over 13 000 secondary metabolites from marine sources have been identified[1] and approximately 300 patents issued[2] on bioactive marine natural products. The majority of these compounds are from sponges, molluscs, byrozoans, and tunicates, but since the mid-1980s mounting evidence has suggested that marine microorganisms, such as bacteria, fungi, and cyanobacteria, might be the true producers of many of the compounds isolated from these invertebrates.[3]

Along with supporting this hypothesis,[3a] investigations into marine cyanobacteria have resulted in many new pharmacologically interesting natural products.[1] Recently, we have been examining the chemical constituents of various strains of *Symploca* spp. (Oscillatoriaceae) collected in Micronesia, and here we report the isolation and structure determination of tasiamide B (**1**) from one of these organisms.



1

The *Symploca* sp. designated NIH304, which had already produced a lipophilic extract, was re-extracted with 30% ethanol in water to afford 2 g of aqueous extract. KB[4] cytotoxicity-guided fractionation of the aqueous extract by Sephadex LH-20 and repeated reversed-phase HPLC afforded 2.6 mg of **1**, in 0.13% yield based on the crude aqueous extract.

Inspection of the proton, carbon, and mass spectral data laid the framework for the structure determination of **1**. The elemental composition of **1** was deduced as $C_{50}H_{74}N_8O_{12}$ (18 units of unsaturations) on the basis of a high-resolution pseudo-molecular ion peak at *m/z* 1001.5347 ($[M + Na]^+$, Δ 2.9 mDa). The proton spectrum of **1** recorded in $CDCl_3$ indicated the existence of two conformers in a ratio of approximately 5:1, but the signals were well enough dispersed to interpret those from the major conformer. The ^{13}C NMR spectrum showed only 46 resonances for the major conformer; therefore, in accord with the mass spectral data, elements of symmetry had to be present in the molecule. Signals in the proton and carbon NMR spectra allowed us to attribute this symmetry to the presence of two monosubstituted phenyl rings, which accounted for eight of the double-bond equivalents. Among the other resonances present in the ^{13}C NMR spectrum were nine carbonyls (δ_C >168), two amide type *N*-methyl groups (δ_H 3.06, 2.82), and two oxygen- and seven nitrogen-bearing methines (δ_C 69.4, 68.7, 59.2, 55.9, 55.6, 54.0, 53.8, 51.9, 45.2).

Further NMR analysis (Table 1) established several large fragments. Alanine, leucine, and valine units were present, while the two *N*-methylamide signals were expanded into *N*-methylphenylalanine and *N*-methylglutamine. Two primary amide proton signals (δ_H 6.52 and 6.37) distinguished this latter possibility from *N*-methylglutamic acid, despite the lack of HMBC correlations to these exchangeable proton signals from the ϵ-carbonyl (C-41). Another unit was assembled starting from the methyl group at δ_H 1.39 (H-50). This was expanded into a lactic acid unit based on a COSY correlation to a quartet at δ_H 4.15 (H-49) that had a $^1J_{CH}$ to one of the oxygen-bearing methines (δ_C 68.7). The second carbinol proton signal (H-28) showed COSY cross-peaks to a methine proton signal at δ_H 4.16 (H-29) and methylene proton signals at δ_H 2.42 and 2.29 (H-27). HMBC correlations to H-27 from C-26, to H-30 from C-29, and to H-30 from C-31 indicated this fragment was 4-amino-3-hydroxy-5-phenylpentanoic acid (Ahppa). Further analysis of the COSY spectrum established an isolated chain (C-2 to C-5) consisting of one methine and three pairs of diastereotopic proton signals. A HMBC correlation to the methine proton (H-2) from a carbonyl at δ_C 172.6 (C-1) established this was an α-amino acid, while the proton and carbon chemical shifts (δ_H 3.42, 3.25, and δ_C 46.9) for the other end of this moiety (C-5) indicated a nitrogen-bearing methylene. Between the two possibilities, either *O*-methylornithine or *O*-methylproline, the first was excluded on the basis of the molecular formula. Thus **1** was composed of the following fragments: Ala, Leu, Val, *N*-Me-Phe, *N*-Me-Gln, lactic acid, Ahppa, and *O*-Me-Pro.

* To whom correspondence should be addressed (R.E.M.) Tel: (808) 956-7232. Fax: (808) 956-5908. E-mail: moore@gold.chem.hawaii.edu.
[†] University of Hawaii at Manoa.
[‡] University of Guam Marine Laboratory.
[§] Present address: The Smithsonian Marine Station, Fort Pierce, FL.

10.1021/np030114z CCC: $25.00
Published on Web 00/00/0000 PAGE EST: 3.4

Table 1. NMR Spectral Data for the Major Conformer of **1** in $CDCl_3$

	C/H no.	δ_H[a] (J in Hz)	δ_C[b,c]	COSY	HMBC[d,e]
O-Me-Pro	1		172.6, s		2, 3, 6
	2	4.43, t (6.7)	59.2, d		3, 4
	3	2.22, m	28.8, t	2, 4	2, 5
		1.83, m		2	
	4	1.91, m	25.3, t		2, 3, 5
		1.81, m			
	5	3.42, ddd (10.9, 6.9, 4.5)	46.9, t	4, 5b	3
		3.25, m		4, 5a	
	6	3.75, s	52.3, q		
N-Me-Phe	7		168.1, s		8
	8	5.63, dd (9.1, 6.7)	55.6, d		9, 16
	9	3.20, dd (−14.8, 6.7)	34.7, t	8	8, 11
		2.95, dd (−14.8, 9.1)			
	10		136.7, s		8
	11/15	7.20, d (7.8)	129.5, d	12/14	
	12/14	7.23, dd (8.0, 7.8)	128.4, d	11/15, 13	
	13	7.18, t (8.0)	126.7, d	12/14	
	16	3.06, s	31.8, q		8
Ala	17		172.4, s		16, 18, 19
	18	4.69, p (7.1)	45.2, d	18-NH, 19	18-NH, 19
	19	0.82, d (7.1)	17.3, q	18	18
	18-NH	6.98, d (7.1)		18	
Leu	20		171.7, s		18-NH, 21
	21	4.32, q (7.9)	51.9, d	21-NH, 22	22
	22	1.48, m	41.0, t	21, 23	21, 23
	23	1.56, m	24.7, d	22, 24, 25	24, 25
	24	0.88, d (6.4)	22.9, q	23	23
	25	0.86, d (6.2)	21.9, q	23	23
	21-NH	7.35, d (7.9)		21	
Ahppa	26		171.6, s		21-NH, 27
	27	2.42, dd (−14.6, 9.4)	40.7, t	28	
		2.29, dd (−14.6, 4.4)			
	28	4.02, ddd (9.4, 4.4, 2.4)	69.4, d	27, 29	27, 29, 30
	29	4.16, dddd (8.9, 7.1, 6.5, 2.4)	53.8, d	28, 29-NH, 30	27, 30
	30	2.94, dd (−14.1, 6.5)	37.2, t	29	32
		2.84, dd (−14.1, 8.9)		29	
	31		137.9, s		30
	32/36	7.20, d (7.8)	129.0, d	33/35	30
	33/35	7.23, dd (8.0, 7.8)	128.2, d	32/36, 34	
	34	7.18, t (8.0)	126.4, d	33/35	
	29-NH	7.10, d (7.1)			
N-Me-Gln	37		169.9, s		29-NH, 38, 39
	38	4.97, m	55.9, d	39	42
	39	2.13, m	22.9, t	38	
		1.83, m			
	40	2.15, m	31.2, t		39
	41		175.5, s		39, 40
	41-NH	6.52, br s			
		6.37, br s			
	42	2.82, s	30.9, q		
Val	43		173.4, s		42, 44
	44	4.47, t (7.6)	54.0, d	45	44-NH, 45, 46, 47
	45	1.94, m	30.4, d		
	46	0.95, d (6.7)	17.8, q	45	44
	47	0.93, d (7.1)	19.3, q	45	44
	44-NH	7.23, d (7.6)		44	
lactic acid	48		176.2, s		44-NH, 49
	49	4.15, q (6.9)	68.7, d	50	50
	50	1.39, d (6.9)	20.8, q	49	49

[a] Recorded at 500 MHz. [b] Recorded at 125 MHz. [c] Multiplicity deduced from HSQC. [d] Protons showing long-range correlation with indicated carbon. [e] If not indicated otherwise, correlations were observed for $^nJ_{CH} = 7$ Hz.

The sequence of these fragments was determined by HMBC and ROESY correlations. Cross-peaks from the secondary amide proton signals to adjacent carbonyls linked the majority of these units into two fragments: Ala-Leu-Ahppa-(*N*-Me-Gln) and Val-lactic acid. These two fragments were joined by a HMBC correlation to the *N*-methylamide signal of the first unit (H-42) from the carboxy terminus of valine (C-43). A similar correlation to the *N*-methylamide signal of *N*-Me-Phe (H-16) appended it to the alanine terminus of this linear chain (C-17), while a ROESY correlation from H-5 of proline to H-8 confirmed the attachment of the final unit, *O*-methylproline, to the carboxy terminus of *N*-methylphenylalanine (C-7).

The absolute configurations of the amino acid-derived units in **1** were determined by chiral HPLC of the hydrolysate. Comparison with authentic standards indicated stereocenters derived from L-Pro, *N*-Me-L-Phe, L-Ala, L-Leu, L-Val, and L-lactic acid. The configuration of the glutamine-derived center was determined by the presence of *N*-Me-L-Glu in the hydrolysate. Ozonolysis of the hydrolysate with oxidative workup provided a peak that co-injected with L-aspartic acid, providing the configuration of C-29.[5] When an attempt to prepare an oxazolidinone derivative by treatment of **1** with triphosgene failed, which would have allowed us to relate the configuration of vicinal centers C-28 and C-29,[6] the (*R*)- and (*S*)-methoxyphenylacetic acid

derivatives were prepared.[7] Unfortunately, the (*R*)-MPA adduct decomposed upon purification over silica and the remaining derivative provided no useful stereochemical information upon the addition of a barium(II) salt[8] or from a variable-temperature NMR experiment.[9] In both cases, any differences in the chemical shifts were determined primarily by an overall conformational change in **1**, which overwhelmed the $\Delta\delta_H$ due to the MPA group.

Even though the stereochemistry of C-28 could not be determined by chemical means, NMR analysis pointed to a 28*S*,29*S* stereochemistry for the Ahppa unit. Inspection of the ^{1}H NMR data in the literature for synthetic 4-amino-3-hydroxy-5-phenylpentanoic acid compounds suggested that the chemical shifts and the coupling constants for the C-2 methylene protons (C-27 in **1**), recorded in $CDCl_3$, were indicative of the C-3/C-4 relative stereochemistry; that is, the downfield methylene proton signal (H-2) generally showed a larger coupling with H-3 when the configuration was 3*S**,4*S** and a smaller coupling when it was 3*R**, 4*S**.[10−12] In our case, the downfield H-27 signal (δ_H 2.42) showed a large proton coupling (J = 9.4 Hz) to H-28, suggesting a 28*S*,29*S* configuration. The same conclusion was reached by a molecular modeling study on the (L-Gln)-Ahppa-(L-Leu) tripeptide fragment (see Supporting Information for figure). Regardless of the stereochemistry of the Ahppa unit, the preferred conformation placed the Ahppa carbonyl (C-26) oxygen and the secondary amide proton (29-NH) on the same face on the molecule where both were hydrogen bonded to the secondary alcohol group on C-28. These results implied that the conformation of the Ahppa unit was locked.[13] Assuming that this hydrogen bonding was occurring in **1**, then only a 28*S*,29*S* stereochemistry was consistent with the proton−proton coupling constants and all the ROESY correlations observed for the protons in the Ahppa unit. Specifically cross-peaks were observed between H-27$_u$/H-29, H-27$_u$/H-28, H-28/H-32, H-29/H-36, and H-28/H-30$_u$. Low-energy conformers could not be found for the tripeptide fragment containing a 28*R*,29*S*-Ahppa unit that were in agreement with the proton−proton coupling constants and the observed NOE correlations. On the basis of these considerations we tentatively suggest an *S*-configuration for C-28.

This new metabolite is similar to another compound isolated from the lipophilic extract of this collection, tasiamide A [(*O*-Me-L-Pro)−(*N*-Me-D-Phe)−(Gly)−(L-Ile)−(*N*-Me-L-Gln)−(L-Leu)−(L-2-hydroxy-3-methylvaleric acid)],[14] and has been assigned a trivial name that reflects this fact. The main difference between tasiamides A and B is the incorporation of an Ahppa unit into **1**, which is the first time this modified amino acid has been found in a metabolite from a marine cyanobacterium.[15] The Ahppa unit is known as a component of protease inhibitors from *Candida*[16] and *Streptomyces*[17] spp. and has also been widely utilized in the field of peptidomimetics. Other δ-amino-β-hydroxyacid-containing metabolites from marine cyanobacteria include valine and proline derivatives in lyngbyabellin D[5] and symplostatin 1,[18] respectively. The incorporation of modified amino acids, formed by a combination of polyketide synthases and nonribosomal peptide synthetases, is a common theme among cyanobacterial metabolites[19] and is one of the reasons investigations into these organisms remain so fruitful.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a Jasco-DIP-700 polarimeter at the sodium D line (589 nm). The UV spectrum was determined on a Hewlett-Packard 8453 spectrophotometer, and the IR spectrum was recorded on a Perkin-Elmer 1600 FTIR instrument as a film on a NaCl disk. MALDI spectra were recorded in the positive mode on a DE-STR spectrometer. The NMR spectra of **1** were recorded in $CDCl_3$ on a Varian 500 operating at 500 and 125 MHz using the residual solvent signal as the internal reference. HPLC separations were performed on a Beckman 110B apparatus coupled to an Applied Biosystems 759A absorbance detector.

Biological Material. The cyanobacterium NIH304 was collected at Short Drop-off in Palau during May 2000. The sample was identified by V. J. Paul, and a voucher is maintained at the Smithsonian Marine Station, Fort Pierce, FL.

Extraction and Isolation. The cyanobacterium (300 g), which had already been extracted with 4:1 $CH_3CN-CH_2Cl_2$,[14] was exhaustively extracted with 30% aqueous EtOH to give 2 g of material. After partitioning between *n*-BuOH and water, the dry organic residue was loaded onto a Sephadex LH-20 column (50 × 2.5 cm) and eluted with 5% MeOH in $CHCl_3$. The fraction eluting between 100 and 140 mL was purified by C_{18} chromatography using increasing amounts of CH_3CN in H_2O. The 50% CH_3CN fraction was separated by RP-HPLC with 45% aqueous CH_3CN (t_R 10.5 min) [Ultracarb ODS, 250 × 10 mm, 3 mL/min, detection at 220 nm]. Final purification with 70% aqueous MeOH [YMC-AQ ODS, 250 × 10 mm, 2 mL/min, detection at 220 nm] provided 2.6 mg of pure **1** (t_R 14.2 min).

Tasiamide B (1): amorphous powder; $[\alpha]^{21}_D$ −28° (c 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 201 (7.60) nm; IR (film) ν_{max} 3396, 3307, 1733, 1633, 1538, 1455, 1265, 1176 cm^{-1}; ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, and HMBC data, see Table 1; MALDI *m/z* [M + Na]$^+$ 1002; HR-MALDI *m/z* [M + Na]$^+$ 1001.5347 (calcd for $C_{50}H_{74}N_8O_{12}Na$ 1001.5318, 2.9 mDa error).

Absolute Stereochemistry of 1. A small portion of **1** (0.3 mg) was dissolved in 0.3 mL of 6 N HCl and refluxed for 24 h at 118 °C. The acid was removed under a stream of N_2 and the residue suspended in 10 mL of methanol. Ozone was then bubbled through this solution for 30 min. The solvent was removed and the residue dissolved in a 1:1 mixture of concentrated formic acid and 30% H_2O_2, stirred overnight, and then refluxed for 1 h at 80 °C.[5] Analysis of the resulting mixture by chiral HPLC [Chirex Phase 3126 (+), 150 × 4.6 mm, flow rate 0.8 mL/min, detection at 254 nm] before and after ozonolysis established stereocenters in **1** derived from L-Asp (t_R 11.9 min), L-Ala (20.1), L-Pro (28.3), L-Leu (32.5), L-Val (37.8), *N*-Me-L-Phe (38.2), L-lactic acid (46.5), and *N*-Me-L-Glu (73.0) by comparison with standards. The retention times of the authentic standards were as follows: with 85:15 CH_3CN−2 mM $CuSO_4$ *N*-Me-L-Phe (38.2) and *N*-Me-D-Phe (42.0); with 90:10 CH_3CN−2 mM $CuSO_4$ L-Leu (32.5), D-Leu (36.8), *N*-Me-D-Glu (36.2), and *N*-Me-L-Glu (73.0); with 95:5 CH_3CN−2 mM $CuSO_4$ L-Asp (11.9) and D-Asp (15.1); with 2 mM $CuSO_4$ L-Pro (28.3), D-Pro (60.2), L-Val (37.8), D-Val (64.3), L-lactic acid (46.5), and D-lactic acid (62.4); with 0.25 mM $CuSO_4$ L-Ala (20.1) and D-Ala (29.0).

Acknowledgment. We thank the National Cancer Institute for providing support through R01 grant CA12623 and NCNPDDG grant CA53001. The upgrades to the spectrometers used in this research were funded by grants from the CRIF Program of the National Science Foundation (CHE-9974921), the Air Force Office of Scientific Research (F49620-01-1-0524), and the Elise Pardee Foundation. The cyanobacterium was collected by S. Belliveau, J. Biggs, I. Kuffner, B. Thacker, E. Cruz-Rivera, and H. Luesch. The KB and LoVo bioassays were performed by G. Tien at the University of Hawaii, Department of Chemistry. The UCR Mass Spectrometry Facility at the Department of Chemistry, University of California at Riverside, performed the mass spectral analyses.

Supporting Information Available: ^{1}H and ^{13}C NMR of **1** along with a figure of (L-Leu)−(28*S*,29*S*-Ahppa)−(L-Gln). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Burgess, J. G.; Wright, P. C. *Tetrahedron* **2001**, *57*, 9347−9377.

(2) Proksch, P.; Edrada, R. A.; Ebel, R. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 125−134.

(3) (a) Luesch, H.; Harrigan, G. G.; Goetz, G.; Horgen, F. D. *Curr. Med. Chem.* **2002**, *9*, 1791−1806. (b) Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2162−2178.

(4) Human epidermoid carcinoma cells.

(5) Ozonolysis of the dehydration product, 4-amino-5-phenyl-2-pentenoic acid, produces phenylalanine, which after prolonged ozonolysis affords aspartic acid. For another example of this approach to the stereochemical determination of γ-amino-β-hydroxyacids see: Williams, P. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2003**, *66*, 595−598.

(6) Insufficient material was isolated to determine the relative configuration of these two centers by a *J*-based approach. Matsumori, N.; Kaneno, D.; Murata, M.; Nakamura, H.; Tachibana, K. *J. Org. Chem.* **1999**, *64*, 866−876.

(7) The adduct of (*R*)-MPA and **1** was formed by DCC coupling and purified by silica chromatography with increasing amounts of ethyl acetate in hexane. The (*S*)-adduct was formed by EDCI coupling. After partitioning between cold 1 N HCl and CH_2Cl_2, the organic layer provided a derivative that was pure enough to be analyzed.

(8) Latypov, S. K.; Seco, J. M.; Quiñoá, E.; Riguera, R. *J. Am. Chem. Soc.* **1998**, *120*, 877−882.

(9) García, R.; Seco, J. M.; Vázquez, S. A.; Quiñoá, E.; Riguera, R. *J. Org. Chem.* **2002**, *67*, 4579−4589.

(10) A similar phenomenon has been observed for β-hydroxy ketones derived from the aldol reactions of chiral aldehydes, see: Roush, W. R.; Bannister, T. D.; Wendt, M. D.; VanNieuwenhze, M. S.; Gustin, D. J.; Dilley, G. J.; Lane, G. C.; Scheidt, K. A.; Smith, W. J., III. *J. Org. Chem.* **2002**, *67*, 4284−4289.

(11) For NMR data on (3*S**,4*S**)-Ahppa: (a) Palomo, C.; Miranda, J. L.; Linden, A. *J. Org. Chem.* **1996**, *61*, 9196−9201. (b) Bonni, B. F.; Comes-Fronchini, M.; Fochi, M. I.; Labonoi, F.; Mazzant, G.; Ricci, A. *J. Org. Chem.* **1999**, *64*, 428−429. (c) Doi, T.; Kokubo, M.; Yamamoto, K.; Takahashi, T. *J. Org. Chem.* **1999**, *64*, 8008−8013. For NMR data on (3*R**,4*S**)-Ahppa and other analogues: Andrés, J. M.; Pedrosa, P.; Pérez, A.; Pérez-Encabo, A. *Tetrahedron* **2001**, *57*, 8521−8530.

(12) Other amino acid-derived 4-amino-3-hydroxyacids do not always follow this trend, although no exceptions were found for the Ahppa unit in acyclic compounds when the methylenes were reported as diastereotopic.

(13) If this hydrogen bonding occurs within the Ahppa unit of **1**, then the concerns outlined in ref 6 regarding the use of NOE correlations to determine the relative stereochemistry of vicinal center that have a small proton−proton coupling would not be valid.

(14) Williams, P. G.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2002**, *65*, 1336−1339.

(15) Metabolites containing analogous *O*-Me-Tyr units have been isolated from marine bacteria SANK70992. Oosumi, H.; Kaneko, l.; Minekura, H.; Takamatsu, Y.; Maruyama, Y.; Kinoshita, T. Jpn. Kokai Tokkyo Koho 95-157406, 1996.

(16) Sato, T.; Shibazaki, M.; Yamaguchi, H.; Abe, K.; Matsumoto, H.; Shimizu, M. *J. Antibiot.* **1994**, *47*, 588−590.

(17) Omura, S.; lmamura, N.; Kawakita, K.; Mori, Y.; Yamazaki, Y.; Masuma, R.; Takahashi, Y.; Tanaka, H.; Huang, L.-Y.; Woodruff, H. B. *J. Antibiot.* **1986**, *39*, 1079−1085.

(18) Harrigan, G. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 655−658.

(19) Gerwick, W. H.; Tan, L. T.; Sitachitta, N. In *The Alkaloids*; Cordell, G. A., Ed.; Academic Press: San Diego, 2001; Vol. 57, pp 75−184.

NP030114Z