Structure of Amino Acids Isolated from Hydrolyzed HV-Toxin M, a Host-specific Toxin-related Compound Produced by *Helminthosporium victoriae*[†]

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The chemistry of several of the specific toxins isolated from a culture medium of the phytopathogenic fungus *Helminthosporium victoriae*, a causal agent of Victoria blight disease of oat, was studied. The structures of the amino acid components of the isolated HV-toxin M were identified, and the absolute configurations of the asymmetric α -carbons were elucidated as (*S*) (I ~ V, Fig. 1), (*S*) for C-3 of 3-OH-leucine (II), (*R*) for C-3 of 3-OH-lysine (V) and (*Z*) for 3-chlorodehydroalanine (VII).

The structure of the other toxin simultaneously isolated, HV-toxin H, was found to be identical with victorin C.

The phytopathogenic fungus *Helminthosporium victoriae*, a causal agent of Victoria blight disease in a susceptible oat variety, produces host-specific damage to susceptible varieties.^{1,2)} Recently, Macko *et al.*²⁾ and Gloer *et al.*³⁾ have reported structural studies on victorin C, the major component of the host-specific pathotoxin complex, in which its six structural components (five amino acids and glyoxylic acid) were identified, and also presented the primary structure of victorin C.⁴⁾ The chemical properties and structures of minor components of the toxin have also been reported.⁵⁾ In an independent study, we have isolated two host-specific toxin-related compounds (HV-toxins H and M) from a culture filtrate of *H. victoriae*. Five amino acid were isolated from the hydrolysate of Toxin M and their structures were elucidated, including the absolute configurations of some of its asymmetric carbon atoms, using HR-MS, NMR and ORD spectra (I ~ V, Fig.

Fig. 1. Structures of Amino Acid Components of Toxin M and Their Derivatives.

[†] Part I in this series.

1), as has been reported in a preliminary note.⁶⁾ In this paper, we describe details of the physical properties and the process for structural assignments of the amino acid components of Toxin M, and some additional properties of Toxin H. Details of the structural assignment of Toxin M using FAB-MS/MS⁷⁾ will be presented in a coming paper (Part II in this series).

Results and Discussion

Spores of *H. victoriae* were grown by still culture for two weeks at 20°C using Fries' medium supplemented with 1.5% oat flakes. Two toxins H and M were separated by BuOH extraction, repeated column chromatography on LH-20 and Shodex HW-40 columns, and preparative silica gel TLC, and were obtained as separate single spots (silica gel TLC, BuOH–AcOH–H₂O 4:1:1; Toxin H, *R_f* 0.28; Toxin M, *R_f* 0.07). Toxin M showed lower inhibitory activity in a dark CO₂ fixation bioassay, and was obtained in a larger quantity (5 mg/l) than the more active Toxin H (0.5 mg/l).

Toxin M gave (M+H)⁺ at *m/z* 798 from FAB-MS. The free amino groups in Toxin M were selectively acetylated in MeOH with acetic anhydride,⁸⁾ and the products were separated by preparative silica gel TLC. The major component of the acetyl derivative of Toxin M was analyzed by MS (FAB- and SI-MS) and NMR to show two *N*-acetyl groups in its NMR spectra, and gave (M+H)⁺ at *m/z* 882 in its FAB- and SI-MS spectra. These results suggested the presence of two free amino groups in the Toxin M molecule. The UV spectrum showed λ_{max} (MeOH) nm (ϵ): 244 (18,000). The molecular formula for Toxin M was found to be C₃₁H₄₆N₇O₁₁Cl₃ by HR-FAB-MS of the methylation product of the Toxin M diacetate (with CH₂N₂, in MeOH; corresponding to the conversion of VI to VIII in Fig. 1), which showed the molecular ion at *m/z* 910.2894 (calcd. 910.2923).

The isolated Toxin M diacetate was hydrolyzed with 2N HCl for 20 hr at 110°C. The

hydrolysate was concentrated and separated on an Avicel column (BuOH–AcOH–H₂O, 4:1:5, upper phase). Among the five amino acids isolated, I, II, III and V (Fig. 1) were found to be identical with those that have been isolated from a hydrolysate of victorin C,^{2,3)} as was confirmed by HR-MS, NMR and FAB-MS/MS (a discussion of the results will appear in Part II in this series), and the remaining one was glycine (IV, Fig. 1), as was ascertained by TLC and NMR (see the experimental section for details).

The ¹H- and ¹³C-NMR spectra of the methylated product of the Toxin M diacetate (VIII in Fig. 1) showed two methyl groups incorporated in its molecule. NMR chemical shifts of one of them (δ_{H} , 3.78; δ_{C} , 53.4) suggested that a free carboxylic acid group was methylated, and those of the other (δ_{H} , 3.22; δ_{C} , 34.9), that one imino group was methylated (VIII, Fig. 1). The ¹³C-NMR spectrum spectrum included all 23 carbon signals expected from the five amino acid components isolated (I~V, Fig. 1), but also contained a group of an ambiguous number of carbonyl carbon signals around 170 ppm. In addition to these signals, three extraneous signals considered to belong to unknown structural parts were observed at 134.2 (d), 138.0 (s) and 162.0 ppm (s). The unsaturation degree of Toxin M based on its molecular formula is 11, among which 8 is assigned to that of the five known components, and 2 is attributed to a carbonyl and an olefinic group indicated by the chemical shifts of the extraneous signals. The remaining one is explainable by assuming one cyclic function. The partial structure of the unknown part was therefore deduced to be VI (Fig. 1), identical with what had been observed earlier for victorin C.²⁾ The molecular formula of Toxin M containing six amino acids was thus confirmed. The assignments of the sequence of these amino acid using FAB-MS/MS will be discussed in Part II in this series.

To estimate of the stereochemistry of the α -carbon atoms of the four optically active amino acids, each of their 2,4-dinitrophenyl (DNP) derivatives was examined by ORD

Table I. COMPARISON OF ORD AND CD SPECTRAL VALUES FOR OPTICALLY ACTIVE AMINO ACID COMPONENTS OF TOXIN M AFTER CONVERSION WITH DNP AND FOR AUTHENTIC SPECIMENS

ORD 220 nm ($[\Phi]$, MeOH)		CD ($\Delta\epsilon$, MeOH)	
DNP-Cl ₂ -Leucine	+14,400		
DNP-OH-Leucine	+19,600		
DiDNP-OH-Lysine	> + 9,300	> -0.14 (432 nm)	> +0.24 (355 nm)
DiDNP-UVF	> + 3,000	> -0.10 (425 nm)	> +0.13 (360 nm)
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(L)-DNP-Leucine ^b	+15,200 ^a		
(L)-DNP-Threonine	+32,300 ^{a,b}		
(L)-DiDNP-Lysine	+30,000 ^a	-0.15 (410 nm)	+0.24 (350 nm)
(L)-DiDNP-Tyrosine	+ 8,200 ^a	-0.27 (410 nm)	+0.47 (338 nm)

^a See ref. 9.^b In 4% Na₂CO₃.**Table II.** COMPARISON OF CHEMICAL SHIFTS FOR 3-HYDROXYLYSINE AND 3-HYDROXYLEUCINE IN ¹H-NMR (ppm/D₂O)

Origin			H-2	H-3	H-6	H-4,5	OMe
3-OH-Lys	COOH	Victorin C ^a	4.08	4.24	3.06	1.5~2.0	—
		HV-Toxin M	4.06	4.22	3.05	1.64~1.89	—
	COOMe	<i>erythro</i> -DiHCl ^b	4.10	3.94	2.90	1.67	3.70
		Toxin M (3N DCl)	4.26	4.32	3.13	1.80	3.77
		<i>threo</i> -DiHCl ^b	4.32	4.25	(—)	1.76	3.86
		Toxin M (D ₂ O)	3.65	4.04	3.05	1.74	3.74
			H-2	H-3	H-4	H-5,5'	
	3-OH-Leu	COOH					
		Victorin C ^c	4.31	3.56	2.01	0.98 / 0.99	
		HV-Toxin M	4.23	3.56	2.02	1.00 / 1.01	

^a *threo*, see ref. 2.^b Synthesized, see ref. 12.^c *erythro*, see ref. 2.

(—), not reported.

[Table I; ORD spectral data of (L)-DNP-leucine to (L)-diDNP-tyrosine were taken from Nagai *et al.*⁹⁾. It is known that many L-amino acids, including those which contain an optically active β -carbon atom beside the active α -carbon atom (e.g., L-threonine and L-isoleucine) exhibit a positive rotation in the same wavelength region.⁹⁾ It seems, therefore, fairly reasonable to assume that the polarity of rotation is in general not much affected by the presence of an additional asymmetry in the β -carbon atom, and hence an (*S*)-configuration was assigned to α -carbon atoms of the opti-

cally active components (**I**, **II**, **III** and **V**, Fig. 1) of Toxin M. The stereochemistry of C-3 in the obtained 3-hydroxyleucine was elucidated as (*S*)-configuration, based on a comparison of $[\alpha]_D$ values [obsd., $[\alpha]_D^{25} = +15^\circ$ ($c=0.22$, H₂O); authentic (2*S*, 3*S*)-3-hydroxyleucine, $[\alpha]_D = +25^\circ$ ($c=1.05$, H₂O)¹⁰⁾; (2*S*, 3*R*)-3-hydroxyleucine, $[\alpha]_D = -3.5^\circ$ ($c=2$, H₂O)¹¹⁾], and NOE measurements of the methylated product of Toxin M diacetate (details will be discussed in Part II) suggested an *erythro*-configuration between H-2 and H-3, as with the case of victorin C (Table II).^{2,3)} The stereo-

chemistry of C-3 in the obtained 3-hydroxylysine was elucidated as (*R*) by comparing the ^1H -NMR chemical shifts of the free amino acid and of its methylated dihydrochloride salt obtained from Toxin M with *threo*-3-hydroxylysine methyl ester dihydrochloride obtained by synthesis¹²⁾ and *threo*-3-hydroxylysine obtained from victorin C.²⁾ The results of ^1H -NMR spectra are summarized in Table II.^{2,12)} The stereochemistry of the exocyclic double bond in **VI** was estimated as (*Z*)-configuration, based on the following conjecture. Firstly, the chemical shift of its olefinic proton was in a fairly low field (δ_{H} 7.68) and was closer to that of the (*E*)-3-chloro- (H-3, δ_{H} 7.32) than to that of the (*Z*)-3-chloro-acrylic acids (H-3, δ_{H} 6.85).¹³⁾ Secondly, when compared to the olefinic proton signals of the (*Z*)-OMe and (*E*)-OMe derivatives of Toxin M (δ_{H} 7.55 and 6.78, respectively), it was closer to that of the (*Z*)-OMe compound.

An amino acid analysis of the acid hydrolysates of Toxin M gave five major peaks of amino acids with the following approximate retention times: 48 min (**III**; authentic aspartic acid, 50 min), 100 min (**IV**; glycine), 131 min (**II**; valine, 129 min), 185 min (**I**; glucosamine, 195 min) and 222 min (**V**; ornithine, 227 min). A hydrolysate of Toxin H gave four amino acid peaks having respective retention times identical with those of **III**, **II**, **I** and **V**.

N-acetylation of Toxin H gave the *N*-monoacetylated Toxin H, indicating the presence of one free amino group. The ^{13}C -NMR spectrum of the *N*-acetyl derivative showed signals with close resemblance to those of victorin C; especially, the three signals due to the amino acid **VI** [at 128.7 (d), 133.0 (s) and 162.4 ppm (s)], and one signal due to glyoxylic acid [at 94.3 ppm (d)] suggested common amino acid constituents for Toxin H and victorin C. Furthermore, the high biological activity of Toxin H also supported its identity as victorin C.

Previously, we have isolated victoxinine- α -glycerophosphate (VGP)¹⁴⁾ from low-activity cultures of *H. victoriae* in a medium without added oat flakes,^{15,16)} and considered this as a

possible component of HV-toxin, as has been earlier considered for victoxinine.¹⁷⁾ Actually, the acid hydrolysates of HV-toxins H and M did not give these spots on TLC, and it was made clear that HV-toxins did not contain VGP or victoxinine as their constituent, even though both VGP and victoxinine were detected in the high-activity culture supplemented with oat flakes.

The biological activities of Toxin M and of Toxin H isolated along with M were preliminarily investigated by the dark CO_2 fixation method. In this bioassay, Toxin H showed a 50% inhibition below 20 ng/ml, and Toxin M showed a 50% inhibition at around 1 ~ 2 $\mu\text{g}/\text{ml}$ on susceptible oat leaves, but no effect was observed on resistant oat leaves at a 1 ~ 2 $\mu\text{g}/\text{ml}$ concentration.⁶⁾

Details of the structural assignments of Toxin M will be presented and discussed in Part II of this series.

Experimental

Analytical methods. Standard analytical methods were carried out using the following instruments: JEOL HX-100 for FAB-MS, FAB-MS/MS and HR-FAB-MS; Hitachi RMU-6M(G) for low-resolution MS, Hitachi M-80 for high-resolution and FD-MS; and JEOL FX-90Q (90 MHz, 22.5 MHz) and JEOL FX- and GX-400 (400 MHz) for ^1H - and ^{13}C -NMR. Perkin-Elmer Model-241 MC polarimeter was used for $[\alpha]_{\text{D}}$ measurements, and Shimadzu IR-435 and Shimadzu UV-200 for IR and UV spectra. The amino acids analysis was carried out on a JEOL-6AH or Hitachi 835. Thin-layer chromatography was carried out on Merck silica gel 60F₂₅₄ (0.25 or 0.5 mm thickness) or on Merck cellulose F₂₅₄ (0.1 mm). The microcrystalline cellulose for column chromatography was Avicel (Funakoshi Pharm. Co., Ltd.).

Cultivation and isolation procedures. Spores of *H. victoriae*, which was suspended in 1% Triton X-100, were grown by still culture for two weeks at 20°C using Fries' medium supplemented with 1.5% oat flakes. Methanol (2 l) and *n*-BuOH (2 l) were added to the 5 l culture filtrate (pH 3.2 ~ 3.4) to precipitate insoluble materials originating from the oat flakes, which was then filtered off. The filtrate was concentrated *in vacuo* to approximately one liter, and extracted with 2 l *n*-BuOH three times. The extracts were combined and concentrated *in vacuo* to give about 12.3 g of crude materials. A portion (2 ~ 3 g) of the crude oily materials was dissolved in 40 ml of methanol, and then

subjected to gel filtration column chromatography (LH-20, 5×75 cm) using methanol as a solvent. The earlier eluates, which were monitored by silica gel TLC using *n*-BuOH-AcOH-H₂O (4:1:1) as the solvent, gave UV-absorbing spots at around 0.07 and 0.28. The last spot had a slightly lower *R_f* than victoxinine- α -glycerophosphate (VGP), which gave a blue spot by spraying 5% vanillin-95% conc. H₂SO₄ and heating. The eluate (240 mg) was subjected to gel filtration column chromatography with a Shodex HW-50, using methanol as the solvent. The eluate (48 mg) was separated by preparative silica gel TLC using *n*-BuOH-AcOH-H₂O (4:1:1 or 4:1:2) as the solvent, and two spots were respectively eluted by the same solvent mixtures. Each of the eluates gave a single spot on silica gel TLC (*n*-BuOH-AcOH-H₂O, 4:1:1), with *R_f* values of 0.28 (corresponding to Toxin H) and 0.07 (Toxin M). Each of the eluates of these spots was further purified by passing through a gel filtration HW-40 column (*n*-BuOH-AcOH-H₂O, 4:1:1), LH-20 (*n*-BuOH-AcOH-H₂O, 4:1:2) and HW-40 (*n*-BuOH-AcOH-H₂O, 4:1:2), and finally gave purified samples of the toxins. The yields from one liter of culture filtrate were about 0.5 mg of Toxin H and 5 mg of Toxin M.

¹³C-NMR spectrum (22.5 MHz, DMSO-*d*₆, ppm) of HV-toxin M. 15.6 (q), 18.3 (q), 19.1 (q), 24.1 (t), 24.9 (t), 31.4 (t), 33.3 (d), 35.9 (t $\times$ 2), 40.8 (t $\times$ 2), 41.5 (d), 50.4 (d), 52.0 (d), 55.2 (d), 59.6 (d), 71.0 (d), 71.5 (d), 81.0 (d), 86.3 (d), 114.3 (s), 132.0 (d), 143.3 (s), 162.8 (s), 173.0 (s), 173.9 (s), 176.2 (s), 185.2 (s) and 205.9 (s); two of the five carbonyl signals of amido groups overlapped with several signals between 173.0 to 176.2 ppm.

Acetylation of the free amino groups of HV-toxins H and M. Each of the HV-toxins H and M (approximately 1 to 2 mg) was dissolved in 4 ml of MeOH to which had been added 1 ml of acetic anhydride, and the solution was stirred at room temperature for four hours, before being kept at 4°C overnight and then concentrated *in vacuo*. Each of the *N*-acetylated toxins was purified by preparative silica gel TLC (*n*-BuOH-AcOH-H₂O, 4:1:1) and column chromatography with LH-20 (MeOH) and HW-40 (MeOH). The *N*-acetylated Toxins H and M gave respective UV-absorbing spots at *R_f* 0.40 and 0.28 on silica gel TLC (Merck F₂₅₄) using the same solvent systems already described.

Properties of N-acetyl-HV-toxin H. UV $\lambda_{\max}$ (MeOH) nm (ϵ): 251 (13,000). ¹³C-NMR (22.5 MHz, CD₃OD) ppm: 14.2 (q), 18.0 (q), 19.5 (q), 22.6 (q, Ac), 25.3 (t), 25.6 (t), 30.0 (d), 31.0 (t), 32.5 (t), 35.0 (t), 39.5 (t), 41.0 (d), 52.1 (d), 53.2 (d), 55.2 (d), 58.3 (d), 70.9 (d $\times$ 2), 79.3 (d), 85.0 (d), 94.3 (d), 114.6 (s), 128.7 (d), 133.0 (s), 162.4 (s), 169.4 (s), 171.4 (s), 172.4 (s), 172.9 (s), 176.8 (s), 178.3 (s), 187.3 (s) and 206.8 (s).

Properties of N,N'-diacetyl-HV-toxin M. FAB- and SI-

MS (glycerol): (M+H)⁺ *m/z* 882. FD-MS of Toxin M: *m/z* 903 (sodium salt). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 244 (18,000). $[\alpha]_D^{22} = -25^\circ$ ($c=0.21$, MeOH). IR $\nu_{\max}$ cm⁻¹ (film): 3400, 3300, 2960, 1655, 1610, 1545, 1430, 1380, 1310, 1260, 1111 and 1080. ¹³C-NMR (22.5 MHz, CD₃OD) ppm: 14.2 (q), 18.5 (q), 19.9 (q), 22.4 (q, Ac), 22.5 (q, Ac), 25.6 (t), 26.4 (t), 30.3 (d), 31.7 (t), 33.1 (t), 35.2 (t), 40.1 (t), 41.5 (d), 43.9 (t), 52.6 (d), 53.7 (d), 55.7 (d), 59.3 (d), 71.5 (d $\times$ 2), 79.9 (d), 85.0 (d), 114.6 (s), 128.7 (d), 133.0 (s), 163.0 (s), 169.9 (s), 170.9 (s), 171.9 (s), 172.8 (s), 173.9 (s), 175.9 (s), 186.3 (s) and 206.8 (s).

Isolation of amino acids from the acid hydrolysate of Toxin M diacetate. Toxin M diacetate (30 mg) was hydrolyzed with 2N HCl for 20 hr at 120°C. The hydrolysate was chromatographically separated using an Avicel column with *n*-BuOH-AcOH-H₂O (4:1:5, upper phase). The fractionated eluates were analyzed using cellulose TLC with *n*-BuOH-AcOH-H₂O (4:1:2), and visualized by spraying ninhydrin solution. The fractions that gave common amino acid spots with respective *R_f* values were combined and concentrated *in vacuo* to give the five amino acids.

Identification of 5,5-dichloroleucine (I). The amino acid obtained from the first eluate of the Avicel column (4.6 mg) showed a retention time of 185 min on an amino acid analyzer column, that of authentic glucosamine being 195 min. *R_f* values on cellulose TLC were 0.80 and 0.84 when the developing solvent was *n*-BuOH-AcOH-H₂O, 4:1:1 and 4:1:2, respectively. SI-MS *m/z* 200 [(M+H)⁺]. ¹H-NMR (90 MHz, D₂O, TSP) δ : 1.23 (3H, d, *J*=7 Hz), 2.20 (2H, m), 2.23 (H, m), 4.18 (H, dd, *J*=11, 6 Hz) and 6.11 (H, d, *J*=3 Hz). ¹³C-NMR (22.5 MHz, D₂O, TSP) ppm: 14.1 (q), 32.7 (t), 39.7 (d), 51.0 (d), 77.8 (d) and 171.7 (s).

The amino acid was refluxed for 2 hr in MeOH-SOCl₂ for methylation of its free carboxylic group. The methyl ester of the amino acid was acetylated (*N*-acetate) with pyridine-Ac₂O (2 ml/0.5 ml, 4°C overnight). The solution was concentrated *in vacuo* to give an *N*-acetyl-methyl ester (**Ia**) having the molecular formula C₉H₁₅NO₃Cl₂. HR-EI-MS *m/z*: obs. 256.0516 [(M+H)⁺, ³⁵Cl], calcd. 256.0508 (³⁵Cl); and *m/z*: obs. 258.0414 (³⁷Cl), calcd. 258.0477 (³⁷Cl). FD-MS *m/z*: 256 [(M+H)⁺]. UV: end absorption. $[\alpha]_D^{22} = -19^\circ$ ($c=0.14$, MeOH). IR $\nu_{\max}$ (film) cm⁻¹: 3280, 3060, 2920, 2840, 1748, 1658, 1540, 1439, 1373, 1265, 1208, 1154, 1004, 963, 838, 747 and 695. ¹H-NMR (400 MHz, CDCl₃, TMS) δ : 1.22 (3H, d, *J*=7 Hz), 1.92 (2H, m), 2.04 (3H, s, Ac), 2.20 (H, m), 3.76 (3H, s, OMe), 4.72 (H, m, CH-N; +D₂O: dd, *J*=8, 5 Hz), 5.80 (H, d, *J*=3 Hz) and 5.96 (H, br. d, *J*=8 Hz; +D₂O, exchangeable). ¹³C-NMR (22.5 MHz, CDCl₃) ppm: 15.2 (q), 22.8 (q, Ac), 35.6 (t), 41.0 (d), 50.0 (d), 52.2 (q, OMe), 78.3 (d), 170.0 (s) and 172.7 (s).

These data on the amino acid and its derivatives confirmed its structure as **I** (Fig. 1), 5,5-dichloroleucine.

Identification of 3-hydroxyisoleucine (II). The structure of the amino acid in the second eluate from the Avicel column (5.5 mg) was identified as **II** (Fig. 1). Its retention time was approximately 131 min on the column of the amino acid analyzer (that of authentic valine was 129 min). Its *R_f* values on TLC were 0.54 and 0.60, respectively, using the two solvent systems described in the foregoing section. SI-MS (glycerol) *m/z*: 148 (*M*+H)⁺. [α]_D²⁵+11° (*c*=0.22, H₂O). ¹H-NMR (90 MHz, D₂O, TSP) δ : 1.00 (3H, d, *J*=7 Hz), 1.01 (3H, d, *J*=7 Hz), 2.02 (H, m), 3.56 (H, dd, CH-O) and 4.23 (H, d, CH-N). ¹³C-NMR (22.5 MHz, D₂O, TSP) ppm: 19.1 (q), 19.5 (q), 31.2 (d), 56.3 (d, CH-N), 77.0 (d, CH-O) and 170.5 (s, COO).

This amino acid was converted similarly to amino acid **I**, to give its *N,O*-diacetyl-methyl ester (**IIa**), molecular formula C₁₁H₁₉NO₅. HR-EI-MS *m/z*: obs. 246.1340 [(*M*+H)⁺], calcd. 246.1311. UV: end absorption. [α]_D²⁵-7° (*c*=0.27, MeOH). IR $\nu_{\max}$ (film) cm⁻¹: 3280, 2960, 2920, 2850, 1746, 1660, 1539, 1435, 1373, 1239 and 1025. ¹H-NMR (400 MHz, CDCl₃) δ : 0.94 (3H, d, *J*=7 Hz), 1.05 (3H, d, *J*=7 Hz), 2.01 (3H, s, Ac), 2.07 (3H, s, Ac), 2.14 (H, m), 3.76 (3H, s, OMe), 4.76 (H, dd, *J*=8, 3 Hz, CH-O), 4.90 (H, dd, *J*=7, 3 Hz, CH-N) and 6.38 (H, br d, NH). ¹³C-NMR (22.5 MHz, CDCl₃) ppm: 18.2 (q), 19.2 (q), 22.8 (q, Ac), 29.5 (d), 52.0 (q, OMe), 53.8 (d, CH-N), 79.6 (d, CH-O) and 171.6 (s, COO).

The data indicated the identity of this amino acid as **II** (Fig. 1), 3-hydroxyisoleucine.

Identification of 3-(2,4-oxy-5-oxo-1-cyclopenten-1-yl)-alanine (III). The amino acid in the third eluate of the Avicel column showed a retention time of approximately 48 min (that of aspartic acid was 50 min) on the amino acid analysis column. Its *R_f* values on TLC were 0.10 and 0.22, respectively, using the foregoing two solvent systems. ¹H-NMR (90 MHz, D₂O, TSP) δ : 2.22 (H, dd, *J*=18, 2 Hz), 2.80 (H, dd, *J*=18, 7 Hz), 2.62 (2H, d, *J*=6 Hz), 3.82 (H, m, CH-N) and 4.42 (H, m, CH-O). ¹³C-NMR (22.5 MHz, D₂O, TSP) ppm: 23.9 (t), 41.9 (t), 55.3 (d, CH-N), 70.2 (d, CH-O), 111.7 (d), 174.9 (s), 201.2 (s) and 202.5 (s).

The amino acid was similarly converted to give its *N,O'*-diacetyl-*O*-methyl-methyl ester (**IIIa**), molecular formula C₁₄H₁₉NO₇. HR-EI-MS *m/z*: obs. 313.1160 (*M*⁺), calcd. 313.1122. UV $\lambda_{\max}$ (MeOH) nm (ϵ): 254 (6,200). IR $\nu_{\max}$ (film) cm⁻¹: 3300, 2920, 2850, 1740, 1660, 1620, 1535, 1435, 1372, 1225, 1175, 1140, 1085, 1030 and 960. ¹H-NMR (400 MHz, CDCl₃) δ : 1.98 (3H, s, Ac), 2.14 (3H, s, Ac), 2.60 (H, dd, *J*=18, 3 Hz), 2.64 (2H, m), 3.28 (H, dd, *J*=18, 6 Hz), 3.71 (3H, s, OMe), 3.97 (3H, s, OMe), 4.56 (H, m, CH-N) and 5.15 (H, dd, *J*=6, 3 Hz, CH-O).

The data indicated the structure of this amino acid as **III** (Fig. 1).

Identification of glycine (IV). The amino acid in the fourth eluate of the Avicel column (3.8 mg) was identified as glycine by an amino acid analysis (a retention time of 100 min, agreeing with that of authentic glycine). Its *R_f*

values were 0.14 and 0.25, respectively, on TLC using the foregoing two solvent systems, identical with those of authentic glycine. ¹H-NMR (90 MHz, D₂O) gave a 2H singlet at δ 3.63, identical with authentic glycine.

Identification of 3-hydroxylysine (V). The amino acid in the fifth eluate of the Avicel column (4.4 mg) showed a retention time of approximately 222 min on the column of the amino acid analyzer (that of authentic ornithine was 227 min). Its *R_f* values on TLC were 0.05 and 0.13, respectively, using the foregoing two solvent systems. SI-MS (glycerol) *m/z*: 163 (*M*+H)⁺. ¹H-NMR (400 MHz, D₂O, TSP) δ : 1.64 (H, m), 1.76 (2H, m), 1.89 (H, m), 3.05 (2H, m, CH₂-N), 3.93 (H, d, *J*=2 Hz, CH-N) and 4.18 (H, m, CH-O). ¹³C-NMR (22.5 MHz, D₂O, TSP) ppm: 24.2 (t), 30.9 (t), 40.3 (t), 58.8 (d, CH-N), 69.3 (d, CH-O) and 171.3 (s, COO).

The amino acid was similarly converted to give its *N,N'*-diacetyl-*O*-acetyl-methyl ester (**Va**), molecular formula C₁₃H₂₂N₂O₆. HR-EI-MS *m/z*: obs. 303.1565 [(*M*+H)⁺], calcd. 303.1555. UV: end absorption. [α]_D²⁵+42° (*c*=0.28, MeOH). IR $\nu_{\max}$ (film) cm⁻¹: 3250, 3040, 2900, 2840, 1753, 1645, 1538, 1425, 1363, 1280, 1223, 1142, 1072, 1040 and 927. ¹H-NMR (90 MHz, CDCl₃) δ : around 1.60 (4H, m), 1.96 (3H, s, Ac), 2.03 (3H, s, Ac), 2.11 (3H, s, Ac), 3.72 (3H, s, OMe), 4.84 (H, dd, *J*=9, 2 Hz, CH-N), 5.23 (H, m, CH-O), 5.88 (H, br. t, ϵ -NH) and 6.22 (H, br d, α -NH). ¹³C-NMR (22.5 MHz, CDCl₃) ppm: 20.4 (q, Ac), 22.8 (q, Ac), 25.0 (t), 28.4 (t), 38.8 (t), 52.3 (q, OMe), 53.7 (d, CH-N), 73.4 (d, CH-O), 169.3 (s) and 169.5 (s, COO).

The evidence indicated the identity of the amino acid as **V** (Fig. 1), 3-hydroxylysine.

Preparation of DNP (2,4-dinitrophenyl) derivatives of the amino acids. Approximately 2 mg of each amino acid was dissolved in one ml of 5% aqueous sodium carbonate containing 10 to 20 mg of FDNB (2,4-dinitro-1-fluorobenzene), and the solution was stirred for 30 min at 40°C.¹⁸⁾ Each amino acid solution was acidified with 6 N HCl and concentrated *in vacuo*. The DNP-derivative from each was purified with repeated preparative silica gel TLC, using CHCl₃-MeOH-AcOH (85:15:1), CHCl₃-MeOH-AcOH (95:5:1) and CHCl₃-MeOH (96:4) as solvents. Their *R_f* values with the solvent system of CHCl₃-MeOH-AcOH (85:15:1) were 0.63 for DNP-5,5-dichloroleucine (**Ib**), 0.45 for DNP-3-hydroxyisoleucine (**IIb**), 0.31 for *N,N'*-diDNP-3-hydroxylysine (**Vb**), 0.08 for *N,O*-diDNP-**III** (**IIIb**), and 0.40 for DNP-glycine.

Properties of *N*-DNP-dichloroleucine (Ib**).** EI-MS of the methyl ester of **Ib** (= **Ic**) gave *m/z*: 379 (*M*⁺, 5%) and 320 (*M*⁺-59, 100%, above *m/z* 300). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 410 (shoulder), 351 (14,200), 260 (6,100) and 215 (10,200). ORD (*c*=0.08, MeOH) [ϕ]_D²² (nm): +14,400 (220). ¹H-NMR (400 MHz, CD₃OD, TMS) δ : 1.20 (3H, d, *J*=7 Hz), 1.99/2.22 (1H, each, m), 2.44 (H, m), 4.27 (H, dd, CH-N), 6.27 (H, d, CH-Cl₂), 7.08 (H, d, DNP's H-6),

8.28 (H, dd, DNP's H-5) and 9.03 (H, d, DNP's H-3).

Properties of N-DNP-3-hydroxyleucine (IIb). EI-MS of a further derivative (IIc) gave m/z : 369 (M^+). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 417 (sh), 354 (13,000), 260 (6,100) and 216 (8,800). ORD ($c=0.19$, MeOH) $[\phi]^{22^\circ}$ (nm): +19,600 (220). $^1\text{H-NMR}$ (400 MHz, CD_3OD , TMS) δ : 1.03 (3H, d, $J=7$ Hz), 1.11 (3H, d, $J=7$ Hz), 2.00 (H, m), 3.62 (H, dd, CH-O), 4.39 (H, d, CH-N), 7.13 (H, d, DNP's H-6), 8.25 (H, dd, DNP's H-5) and 9.03 (H, d, DNP's H-3).

Properties of N,N'-diDNP-3-hydroxylysine (Vb). FD-MS of a further derivative (Vc) gave m/z : 550 (M^+ , 100%) and 551 $[(M+H)^+$, 73%]. UV $\lambda_{\max}$ (MeOH) nm (ϵ): 410 (sh), 350 (17,800), 260 (8,700) and 212. ORD ($c=0.21$, MeOH) $[\phi]^{22^\circ}$ (nm): +7,500 (220). CD ($c=0.21$, MeOH) $\Delta\epsilon$ (nm): -0.14 (432) and +0.42 (355) [the CD spectrum of authentic N,N' -diDNP-lysine ($c=0.16$, MeOH) $\Delta\epsilon$ (nm): -0.15 (410) and +0.24 (350)]. $^1\text{H-NMR}$ (400 MHz, CD_3OD , TMS) δ : 1.43/1.74 (1H each m), 1.74/2.00 (1H each, m), 3.51 (2H, t, $\text{CH}_2\text{-N}$), 4.20 (2H, m, CH-O and CH-N), 7.02/7.08 (1H, each, d/d, DNP's H-6 and H-6'), 8.22/8.25 (1H each, dd/dd, DNP's H-5 and H-5'), 9.05/9.07 (1H each, d/d, DNP's H-3 and H-3').

Properties of O,N-diDNP-III (=IIIb). FD-MS of a further derivative (O' -acetyl- O,N -diDNP-methyl ester, IIIc) gave m/z : 590 $[(M+H)^+$, 2%] and 558 $[(M+H)^+ - 32$, 100%]. UV $\lambda_{\max}$ (MeOH) nm (ϵ): 410 (sh), 353, 265 and 212. ORD ($c=0.18$, MeOH) $[\phi]^{22^\circ}$ (nm): +3,000 (220). CD ($c=0.18$, MeOH) $\Delta\epsilon$ (nm): -0.10 (425) and +0.13 (360) [the CD spectrum of authentic diDNP-tyrosine ($c=0.63$, MeOH) $\Delta\epsilon$ (nm): -0.27 (410) and +0.47 (338)]. $^1\text{H-NMR}$ (400 MHz, CD_3OD , TMS) δ : 2.23/3.21 (1H each, dd, CH_2 in cyclopentenone group), 2.78~2.87 (2H, m), 4.22 (H, CH-N), 4.50 (H, CH-O), 7.10/7.15 (1H each, d/d, DNP's H-6 and H-6'), 8.27/8.29 (1H each, dd/dd, DNP's H-5 and H-5') and 9.04/9.05 (1H each, d/d, DNP's H-3 and H-3').

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