Structure Elucidation of Sch 20562, a Glucosidic Cyclic Dehydropeptide Lactone — the Major Component of W-10 Antifungal Antibiotic

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A novel bacterium designated as *Aeromonas* sp. W-10 produces the antibiotic W-10 complex which comprises of two major and several minor components. The two major components from this complex, Sch 20562 (**1**) and Sch 20561 (**1a**), are of biological interest in view of their potent antifungal activity. The chemical degradation studies utilized for the assignment of structure **1** for Sch 20562 are described here. Some of the noteworthy diversity of structural features in this glucosidic cyclic dehydrononapeptide lactone **1** are: an *N*-terminal (D)-β-hydroxymyristyl unit, three D-amino acid units, two (*E*)-α-amino-crotonyl units, and an *O*-α-D-glucosyl-*N*-methyl-L-*allo*-threonine unit. The structure determination of **1** utilized the selective cleavage of the dehydropeptide units by ozonolysis to form fragments that were sequenced by mass spectrometry. The stereochemistry of the amino acid units were assigned by isolation of the free amino acids from the hydrolysates of the fragments. The stereochemistry of the α-aminocrotonyl units and the glucosidic linkage were assigned by nmr spectroscopy and molecular rotation data.

Antibiotic W-10 complex is produced by a novel bacterium from the genus Aeromonas designated as *Aeromonas* sp. W-10 NRRL B11053. The antibiotic complex is comprised of several components including Sch 20561 and Sch 20562, the two major components in this complex, which are of biological interest due to their potent antifungal activity against yeasts and dermatophytes.[1] We report here the chemical degradation studies that led to the assignment of the structure and stereochemistry **1** for Sch 20562 (Figure 1).[2]

Results and Discussion

The physical and hydrolytic data for Sch 20562 are summarized in Scheme 1.[3] These initial data indicated that Sch 20562 was a macrocyclic glucosidic dehydropeptide lactone wherein the *N*-terminal amine was acylated with a (D)-β-hydroxymyristyl unit (D-Hma). Thus, the amino acid composition and the formation of ammonia and α-keto butyric acid (characterized as 3-ethyl-2-quinoxalinol **3**)[4] in the acid hydrolysate, the high

Fig. 1.   Structure of Sch 20562 (**1**).

D-Hma   D-*allo*-Thr → D-Tyr → *E*-Aca

E-Aca → L-Thr   D-Gln

L-His ← N-Me-L-*allo*-Thr

α-D-Glucopyranosyl(1→3)-O
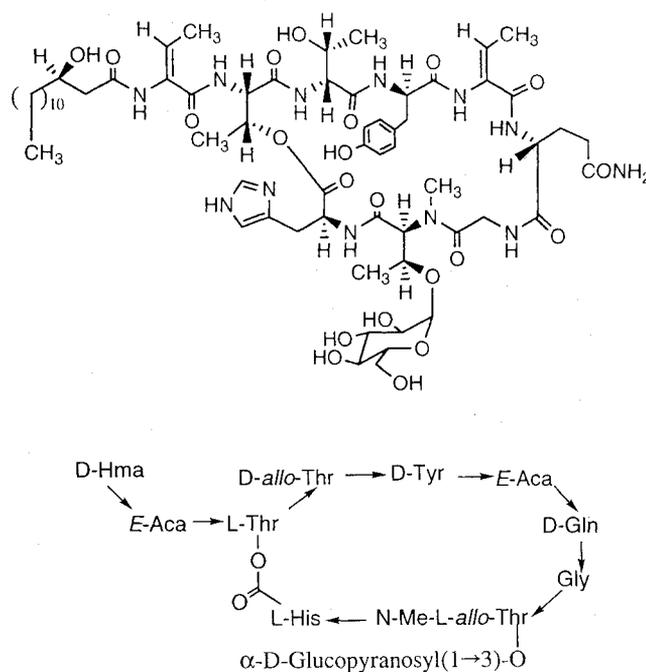
Scheme 1. Physical data and hydrolysis products of 1.

Molecular formula: $C_{63}H_{96}O_{21}N_{12}$
MW: 1356
pKa: 6.5
Ninhydrin reaction: negative
$\lambda_{max}^{MeOH/OH}$ 240 nm (ε 27000), 292 nm (ε 2400)
IR: 1750 cm^{-1} ^{1}H NMR: δ 1.80

$1 \xrightarrow{6N\ HCl}$ His (1), Thr (2), Glu (1), Gly (1), Tyr (1), NH$_3$ (3)

2 → 3

$1 \xrightarrow{NaOH}$ D-Hma (4a)
4a, R = OH
4b, R = OCH$_3$
4c, R = NH$_2$

$1 \xrightarrow{HCl\ /\ MeOH}$ 5

$1 \xrightarrow[Et_3N]{MeOH}$ Methyl ester 6

$1 \xrightarrow{O_3}$ CH$_3$CHO 7 → 8

9
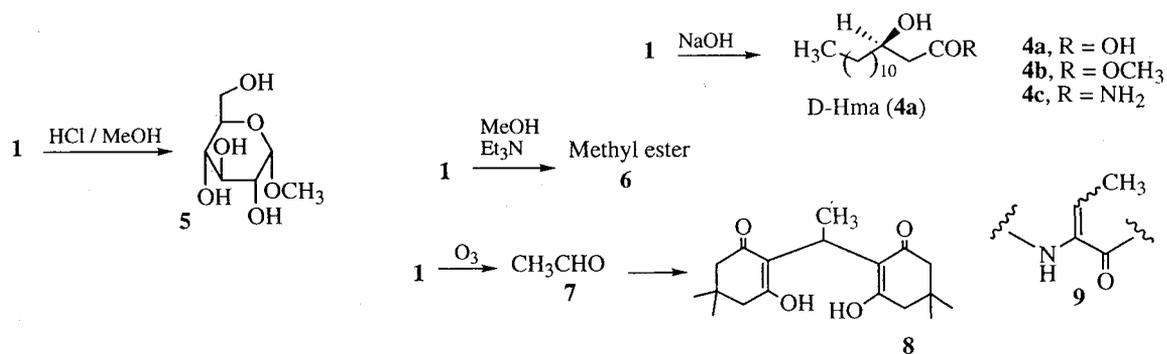
extinction coefficient for the 240 nm absorption in the UV in base[5] and the integration of the olefinic methyl signal in the NMR suggested that 1 was a dehydropeptide containing two α-aminocrotonyl units 9 (Aca). The facile methanolysis of 1 to form a methyl ester 6, the identification of D-Hma (4a)[6] in the base hydrolysis, and 1α-methyl glucoside 5 in the acid catalyzed methanolysis, together with the ninhydrin negative reaction supported a lactonic glucosidic structure with a blocked N-terminal amine for the dehydropeptide. The presence of the dehydro units 9 was further confirmed by the formation of acetaldehyde (characterized as the dimedone adduct 8) in the ozonolysis of 6.

Electrom impact (EI) mass spectrometry of permethylated N-acyl peptides is a convenient technique for the sequencing of oligopeptides. Fragment ions derived from the EI cleavage of the derivatized peptide at the peptide bonds are characteristic since the charge is retained by the N-terminal fragment.[7] We utilized this technique for sequencing the peptides described here. Permethylation of the acyclic methanolysis product 6 afforded 10 which showed the characteristic fragmentation pattern $a \sim g$ in the EI mass spectrum thereby establishing a partial sequence for the peptide 6 (Scheme 2). The fragment ions beyond g were not informative and hence the full sequencing of 6 required smaller fragments derived from this peptide. Mild acid hydrolysis of 1 or 6 to generate

such fragments was found to form complex mixtures of products arising from random peptide fragmentations and this approach was not attractive. At this point, we made use of an observation from the initial experiment in the ozonolysis of 6 to 7 used for confirming the presence of dehydropeptide units 9 (Scheme 1); TLC analysis of the ozonized product showed that 6 was converted into a mixture of only three products which were easily separable in view of their widely different polarities. We reasoned that an intermediate oxalimide 12 formed in the ozonolysis of a dehydropeptide 11 (Scheme 2), would undergo a selective methanolysis at the imide carbonyl to afford the amide 13 and an N-terminal methyloxalamide 14 as the two cleavage products wherein the oxalamide group in 14 and the nitrogen of the amide group of 13 are derived from the dehydro unit.[8] This methodology was utilized to obtain fragments from 6 and from other intermediates derived from 1 that are described here.

Ozonolysis of the acyclic methyl ester 6 followed by reductive work-up with Me$_2$S and methanolysis of the intermediate oxalimide afforded, after chromatography, exclusively the three fragments A $\sim$ C (4c, 15, and 16). The crystalline fragment A was characterized as D-β-hydroxymyristamide 4c. Fragments B and C were sequenced by mass spectrometry of their permethylated derivatives (Scheme 3). Fragment B (15) was a tripeptide

Scheme 2
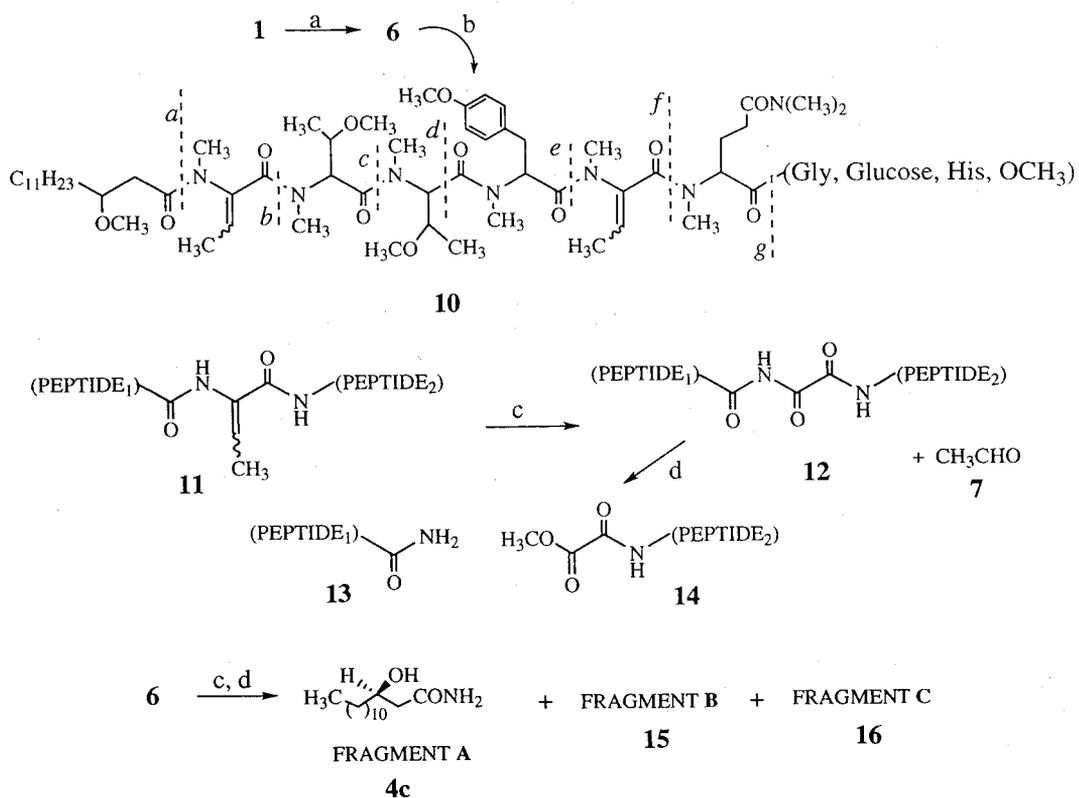
Reagents: (a) MeOH/Et₃N   (b) DMSO/NaH/CH₃I   (c) i. O₃/MeOH/−78°C, ii. Me₂S   (d) Et₃N/rt.

containing the two Thr and the single Tyr units found in **1**, and an *N*-terminal methyloxalyl group derived from one of the Aca units of **1**. The tripeptide reacted with diazomethane to form the methyl ether **15b**, and on acetylation formed the triacetate **15c**. Ammonolysis of **15** afforded the oxalamide **15a**. The sequence in **15** was established by permethylation of **15a** to **17** which showed the fragment ions $a \sim c$ in the EI mass spectrum, in agreement with the amino acid sequence shown.

Fragment C (**16**) was found to be a tetrapeptide containing the remaining residues identified in **1**. Methanolysis of **16** under acidic conditions afforded methyl glucoside, and amino acid analysis of its acid hydrolysate showed, in addition to Glu and Gly, the presence of Asp which was not found in **1**. Tetrapeptide **16** contained an *N*-terminal methyloxalyl group derived from the second Aca unit in **1**, and an *N*-formyl-Asn-OCH₃ as the carboxy terminal unit. The *N*-formyl-Asn unit in the tetrapeptide arises from the ozonolysis of the imidazole ring in the His unit originally present in **1**

and **6**.[9] Acetylation of **16** afforded a tetraacetate **16b**. Additionally, the ¹H NMR spectrum of **16** showed the presence of an N–CH₃ signal at δ 2.90. This information prompted us to use CD₃I in the permethylation of **16** in order to differentiate the methyl group present in **16** from the methyl groups introduced by permethylation. Ammonolysis of the two methyl esters and the *N*-formyl groups of **16** formed the diamide **16a** which was permethylated using CD₃I to afford **18**. The EI mass spectrum of **18** showed the fragment ions $a \sim d$ in agreement with the amino acid sequence shown. The sequence confirmed the presence of an *N*-methyl threonine unit that was not evident in the preliminary hydrolytic composition of **1** because *N*-methyl-amino-acids are not detectable in conventional amino acid analysis based on ninhydrin color yield detection.[10]

In order to ascertain that the Asn residue in **16** (His residue in **1** and **6**) was indeed the terminal carboxy in the acyclic peptide sequence **6** and also the carbonyl of the lactone in **1**, the compound was aminolyzed to **6a**

Scheme 3

Reagents: (a) $NH_3/MeOH$ (b) CH_2N_2/Et_2O (c) Ac_2O/pyr (d) $DMSO/NaH/CH_3I$ (e) $DMSO/NaH/CD_3I$
(f) i. $O_3/MeOH/-78°C$, ii. Me_2S, iii. Et_3N/rt (g) $DMF/EtNH_2$.
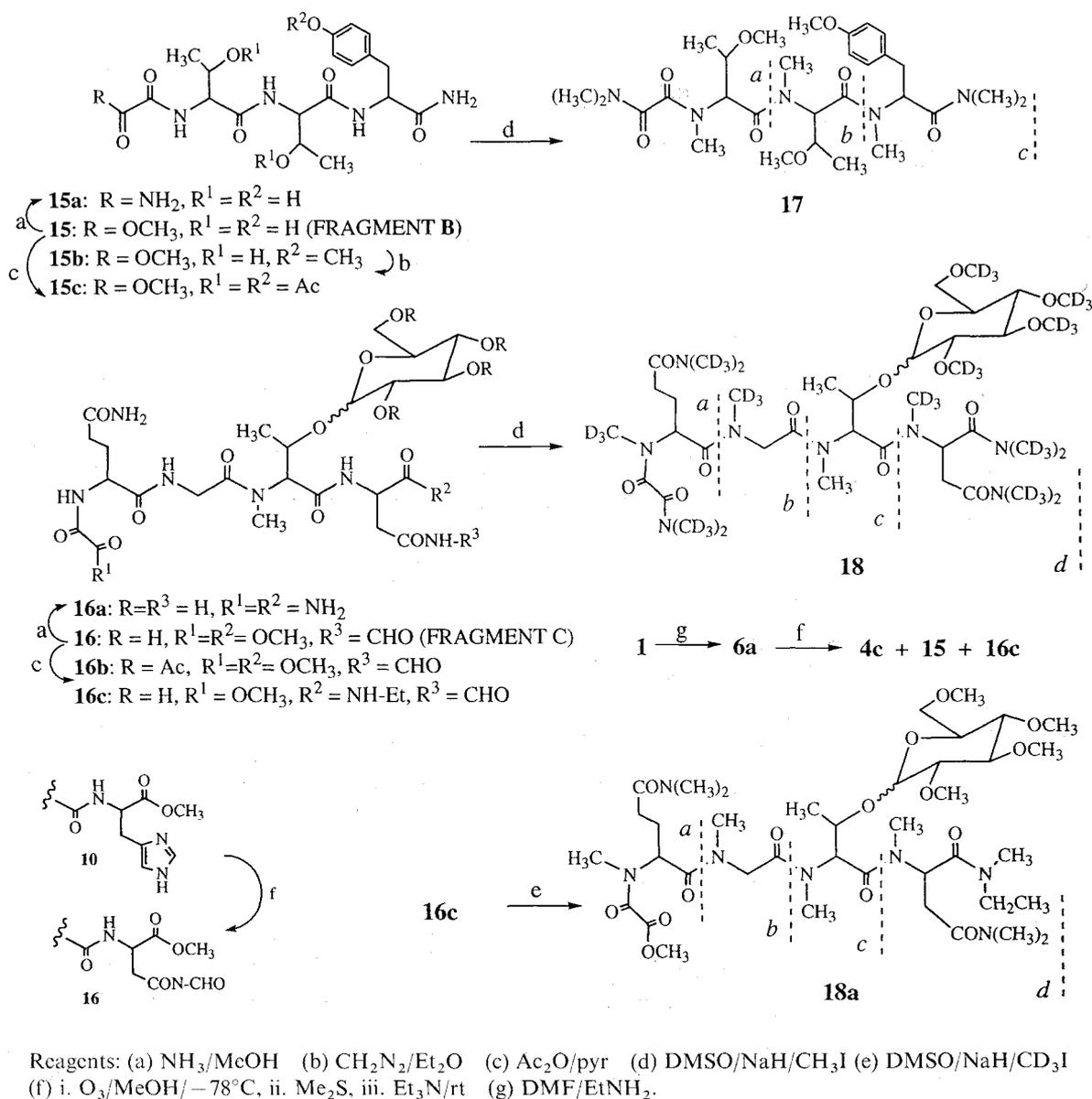
with ethylamine thereby introducing the ethylamide group in **6a** as a tag for the carbonyl of the lactone.[11] Ozonolytic cleavage of **6a** afforded the fragments **4a** and **15** as obtained previously from **6**, and the new tetrapeptide fragment **16c** containing the ethylamide group. The permethylated product **18a** derived from **16c** showed the fragment ions $a \sim d$ in the EI mass spectrum, in agreement with the amino acid sequence shown.

The information provided by the partial sequence **10** in conjunction with the sequence overlapping[12] of the fragments **4c**, **15**, **16**, and **16c** enabled us to assign the full nonapeptide sequence for the acyclic products **6** and

6a derived from **1** (Scheme 4).

We then determined which of three possible hydroxyl groups was involved in the lactonic structure of **1**. This was accomplished by blocking the free hydroxyl groups in **1** by preparing a per-tetrahydropyranyl derivative **19**. Treatment of **19** with aqueous regenerated **1** thereby ensuring that the integrity of the molecule was maintained during the THP formation. Ozonolysis of **19** followed by reductive work-up and methanolysis afforded, after chromatography, the THP derivative of D-β-hydroxymyristamide **4d** as a mixture of diastereoisomers, the tripeptide **20**, and other uncharacterized products. The

Scheme 4

6, R = OCH$_3$
6a, R = NH-Et

1 $\underset{b}{\overset{a}{\rightleftarrows}}$ per-THP derivative **19** $\overset{c}{\searrow}$

20

$\overset{d}{\longrightarrow}$

21

4d

1a
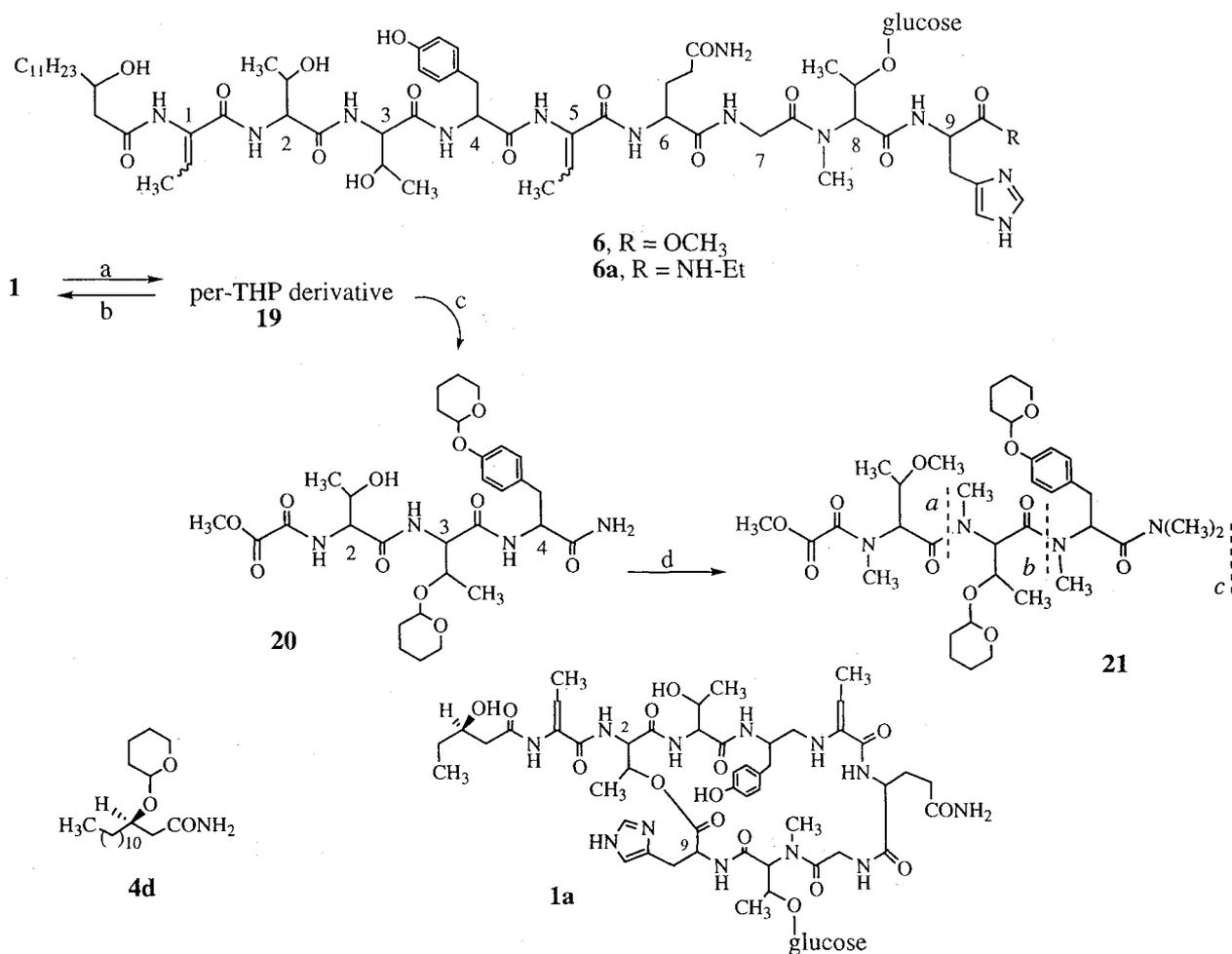
Reagents: (a) DHP/pTSA/DMF   (b) 0.05 N HCl   (c) i. O$_3$/MeOH/ −78°C, ii. Me$_2$S, iii. Et$_3$N/rt
(d) DMSO/NaH/CH$_3$I.

permethylation product **21** derived from **20** showed the fragment ions $a \sim c$ in the EI mass spectrum as expected for the sequence shown. Additionally, the fragment ion at m/e 216 identified the free hydroxyl group in **20** and this result in conjunction with ethylamide functionality in **6a** also established that the hydroxyl group of the threonine-2 and the carboxy of the terminal histidine-9 form the lactonic bond in **1**. The facile methanolysis observed for **1** would be expected for a lactone derived from a histidine unit. The information generated to this point established the gross cyclic structure **1a** for Sch 20562 wherein the only unit with a defined stereochemistry was the N-terminal Hma residue.

Stereochemistry of the Amino Acid Units

The approach used to define the absolute stereochemistry of the amino acid units in **1a** was based on the isolation and characterization of the individual amino acids formed by acid hydrolyses of the tri- and tetrapeptide fragments. Thus, acid hydrolysis of **15** (Scheme 5) afforded D-tyrosine (**22**) and threonine (**23**). However, proton nmr and optical rotation measurements indicated that the threonine **23** isolated from **15** was a 1:1 mixture of L-threonine and D-*allo*-threonine and hence tripeptide fragment required to be modified so as to permit the isolation of the two threonine units separately. This was accomplished by destroying either one of the threonine units by selective oxidation to allow the

Scheme 5
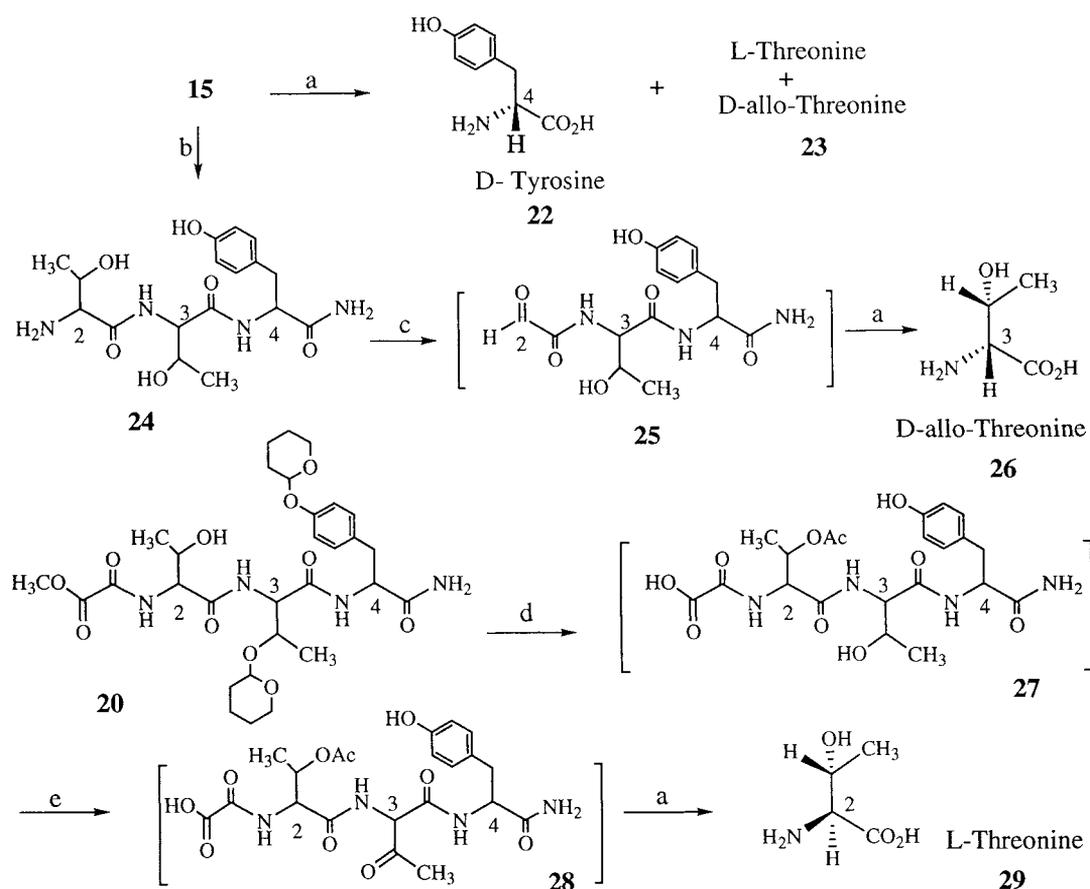
Reagents (a) 6 N HCl/110°C   (b) 1 N HCl/70°C/20 h   (c) NaIO₄/THF/H₂O   (d) ı. Ac₂O/pyridine, ıı. 70%
acetic acid-H₂O   (e) CrO₃-H₂SO₄ (Jones)/Me₂CO.

isolation of the intact threonine unit. The *N*-terminal oxalate group of **15** was hydrolyzed under mild acid conditions and the vicinal amino alcohol of the resulting **24** was oxidized with sodium periodate. The intermediate glyoxylamide intermediate **25**, without purification, was then hydrolyzed to afford the Thr-3 unit which was characterized as D-*allo*-threonine (**26**).

Tripeptide fragment (**20**) was found suitable for accessing the Thr-2 unit. Thus the hydroxyl group of **20** was protected as an acetate followed by removal of the THP groups under mild acidic conditions to afford **27**. The hydroxyl group of the Thr-3 unit in **27** was oxidized with Jones reagent and the resulting mono-threonine derivative **28** was hydrolyzed to afford the Thr-2 unit which was characterized as L-threonine (**29**). Minor chemical modifications of the tripeptide fragments **15** and **20** prior to hydrolysis, thus served to define the

stereochemistry of amino acid units #2, 3, and 4 in **15**, **6**, and **1**.

Hydrolysis of the tetrapeptide **16** afforded D-glutamic acid (**30**), glycine, and L-aspartic acid (**31**) arising from amino acid units #6,7, and 9 (Scheme 6). The hydrolysate did not contain *N*-methyl threonine that is present in the tetrapeptide. We found that the glucosidated hydroxyl in the substrate is prone to *β*-elimination under mild acidic conditions leading to the formation of **2**. Deglucosidation of **16** was hence required prior to hydrolysis in order to isolate the intact *N*-Me-Thr unit for characterization. The glucose group in **16** was oxidized with sodium periodate to afford the expected glyoxal acetal intermediate **32** which was found to be less prone, relative to **16**, to *β*-elimination under mild acidic conditions. Removal of the acetal functionality in **32** was effected under reductive conditions with

Scheme 6
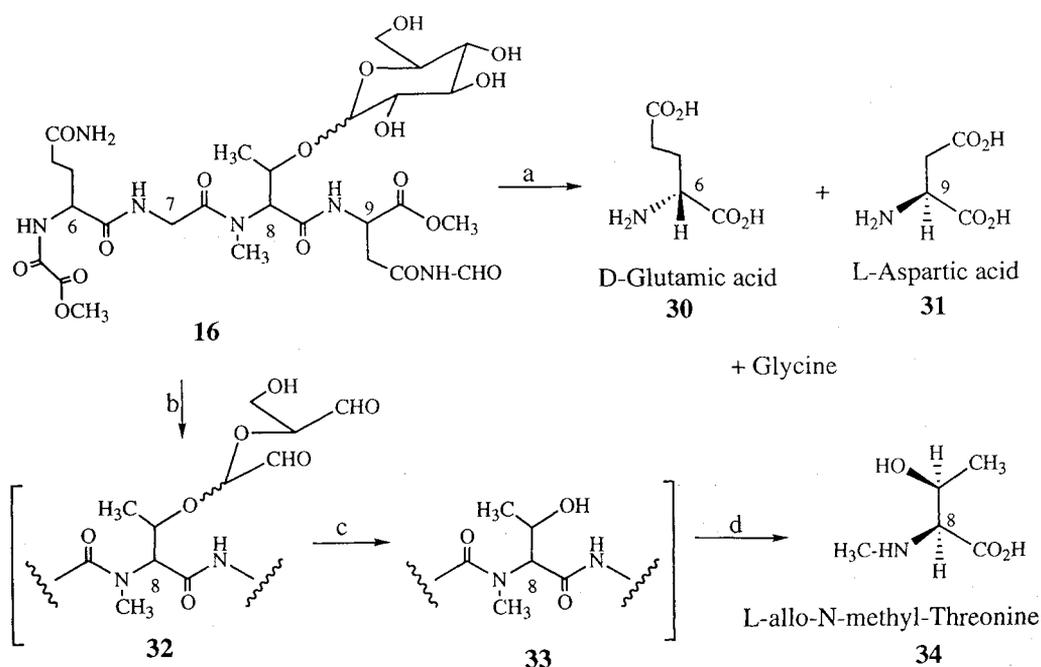
Reagents: (a) i. 6 N HCl/110°C/24 h, ii. silica gel chromatography, iii. CuCO₃/H₂O/separation, iv. H₂S
(b) NaIO₄/H₂O (c) Zn dust/AcOH/H₂O (d) i. 6 N HCl/110°C/24 h, ii. silica gel chromatography.

zinc-acetic acid to provide the unmasked hydroxy compound 33. Compound 33 was found to be stable to β-elimination, and upon hydrolysis afforded the amino acid unit #8 which was characterized as N-methyl-L-*allo*-threonine (34).[13]

Stereochemistry of the Dehydro Units

Assignment of the stereochemistry for 2-acylamino-crotonates (N-acyl-Aca) using NMR spectroscopy has been reported previously. Based on the ¹H chemical shifts in CDCl₃ of several 2-acylaminocrotonates, the isomers with low field resonances for both the methyl doublet and the vinyl quartet were assigned the E configuration.[14] However, we had observed an isomerization of the dehydro unit in the aminolysis of 1 to the ethylamide 6a; the ¹H NMR spectrum of 6a in DMSO-d_6 showed that the vinyl proton was shifted downfield while the olefinic methyl was shifted upfield relative to the resonances in 1,[11] and this suggested that the chemical shift positions and deshielding effects for these protons were solvent dependent.

For direct comparisons of the chemical shifts in

Scheme 7
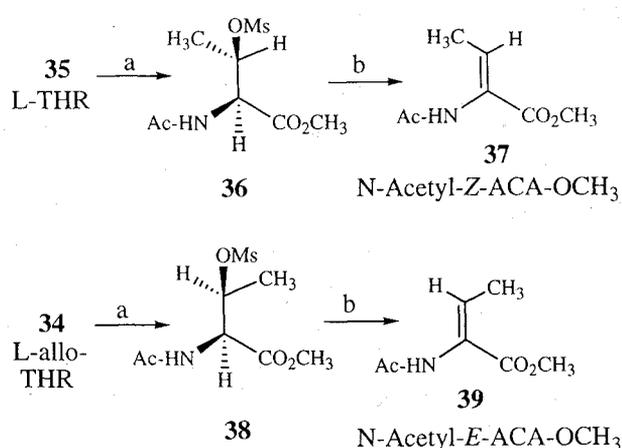
Reagents: (a) i. HCl/AcOH,
ii. NH₄OH, iii. CH₂N₂/Et₂O,
iv. MsCl/Pyr (b) Et₃N/Me₂CO.

DMSO, we prepared the N-acetyl isomers 37 and 39 by the β-elimination of the N-acetyl-O-mesyl-threonine methyl esters 36 and 38 (Scheme 7). It was found that

Table 1. ^{1}H NMR data for dehydro amino acid protons.

Compound	Solvent	Olefinic methyl δ (mult. J Hz)	Vinylic proton δ (mult. J Hz)
Acetyl-Z-Aca-OCH$_3$ (35)	DMSO-d_6	1.70 (d, $J=7$ Hz)	6.48 (q, $J=7$ Hz)
Acetyl-E-Aca-OCH$_3$ (36)	DMSO-d_6	1.82 (d, $J=7$ Hz)	5.90 (q, $J=7$ Hz)
Sch 20562 (1)	DMSO-d_6	1.78 (d, $J=7$ Hz)	5.80 (q, $J=7$ Hz)
		1.82 (d, $J=7$ Hz)	5.84 (q, $J=7$ Hz)
Acetyl-Z-Aca-OCH$_3$ (35)	CDCl$_3$	1.79 (d, $J=7$ Hz)	6.78 (q, $J=7$ Hz)
Acetyl-E-Aca-OCH$_3$ (36)	CDCl$_3$	2.09 (d, $J=7$ Hz)	7.02 (q, $J=7$ Hz)

Chemical shifts in ppm relative to TMS (100 MHz). Assignments are based on decoupling experiments.

Table 2. Glucosidic ^{13}C NMR chemical shifts (ppm).

Compound	C$_1$	C$_2 \sim$ C$_6$
1-OMe-β-D-Glucopyranoside	104.2	61.9 ~ 76.8
1-OMe-β-D-Glucofuranoside	104.2	64.2 ~ 78.8
1-OMe-α-D-Glucopyranoside	100.1	61.7 ~ 72.5
1-OMe-α-D-Glucofuranoside	110.0	64.7 ~ 82.3
Sch 20562	94.9	No signal > 73.4

Chemicaal shifts in (DMSO-d_6 25.2 MHz).

Table 3. Glucosidic contribution to [M]$_D$.

Compound	[M]$_D$ (Solvent)	Δ [M]$_D$
SCH 20562 (1)	$-380°$ (DMSO)	$+228°$
SCH 20561 (1a)[2]	$-608°$ (DMSO)	
16	$+68°$ (H$_2$O)	$+282°$
33	$-214°$ (H$_2$O)	
1-OMe-α-D-Glucoside	$+320°$ (DMSO)	
	$+306°$ (H$_2$O)	
1-OMe-β-D-Glucoside	$-70°$ (DMSO)	
	$-66°$ (H$_2$O)	
1, Calcd. as α-D-Glucoside	$-288°$ (DMSO)	
1, Calcd. as β-D-Glucoside	$-678°$ (DMSO)	
16, Calcd. as α-D-Glucoside	$+92°$ (H$_2$O)	
16, Calcd. as β-D-Glucoside	$-280°$ (H$_2$O)	

either of these substrates afforded a mixture of the olefins 37 and 39 and these could arise by a competing inversion at C$_3$, under the Et$_3$N basic conditions used, followed by trans elimination of the inverted product. ^{1}H NMR data in Table 1 showed that in DMSO-d_6, the vinylic proton in the Z isomer 37 is deshielded by the ester carbonyl by 0.58 ppm relative to the resonance for the E isomer 39 and the methyl doublet in the latter isomer is deshielded by 0.1 ppm relative to the resonance for the Z isomer 37. The chemical shifts for these protons in Sch 20562 (1) correlate with those in 39 and the Aca units were therefore assigned the E configuration.

Stereochemistry of the Glucosidic Linkage

To complete the structure elucidation of Sch 20562, the stereochemistry of the glucosidic linkage at the N-Me-L-$allo$-Thr-8 unit remained to be assigned. Table 2 lists the ^{13}C NMR chemical shifts of anomeric 1-methoxy-D-glucosides and 1. The chemical shift of the anomeric carbon and the absence of signals above δ 73.4

for C$_2$-C$_6$ in 1 was indicative of a α-D-glucopyranoside linkage for glucose linked to the hydroxyl of the N-Me-L-$allo$-Thr-8.

A more definitive assignment was based on the application of optical rotatory behavior of glycosides to configurational studies. It is well established in carbohydrate chemistry that the contribution of the glucosidic linkage to the molecular rotation, [M]$_D$, of a glucosidic compound is characteristic for α-glucosides and β-glucosides.[15] This contribution value can be obtained from the difference, Δ [M]$_D$, between the [M]$_D$'s of the glucoside and its aglycone and the value is equal to the molecular rotation of the corresponding 1-methyl-glucosides. Conversely, the calculated value of the [M]$_D$ of a glucoside corresponds to the sum of the [M]$_D$'s of the aglycone and the corresponding 1-methyl-glucosides.

Table 4. ^{13}C NMR chemical shift assignments for Sch 20562 (1).

Unit	Atom	δ^a	Unit	Atom	δ^a
D-Hma	C_2	43.8	D-Tyr	C_2	54.7
	C_3	67.5		C_3	36.3
	C_4	37.0		$C_{1'}$	128.1
	C_{5-9}	29.1		$C_{2'}$	130.3
	C_{10}	31.3		$C_{3'}$	115.1
	C_{11}	28.7		$C_{4'}$	156.0
	C_{12}	25.0			
	C_{13}	22.1	Gly	C_2	NA^b
	C_{14}	13.9			
			N-Me-L-allo-Thr	C_2	52.4
E-Aca	C_2	NA^b		C_3	71.3
	C_3	121.8		C_4	15.2
		122.5		N-CH$_3$	31.5/32.0
	C_4	13.2			
			L-His	C_2	52.4
L-Thr	C_2	59.2		C_3	NA^b
	C_3	71.4		$C_{2'}$	135.5
	C_4	15.9		$C_{4'}$	115.1
				$C_{5'}$	131.3
D-allo-Thr	C_2	60.8			
	C_3	67.5	α-D-Glucose	C_1	95.0
	C_4	19.6		C_2	71.6
				C_3	73.5
D-Gln	C_2	54.5		$C_{4,5}$	70.1, 70.5
	C_3	26.9		C_6	60.8
	C_4	32.0/31.5			

^{a} Chemical shifts in (DMSO-d_6 25.2 MHz).
^{b} These carbon atoms have not been assigned.

We utilized Sch 20561 (1a)[2] which was found to be the aglycone of 1, and compound 33 which was the aglycone derived from the glucosidic tetrapeptide fragment 16, to determine the glucosidic contribution to the $[M]_D$'s in 1 and 16. The data in Table 3 show that the values fo the Δ $[M]_D$'s for both 1 and 16, are close to the $[M]_D$ value for 1-OMe-α-D-glucoside, and additionally the calculated $[M]_D$ values for 1 and 16 as α-D-glucosides are close to the found values for both the compounds. The glucose linkage at the N-Me-L-allo-Thr-8 unit was hence assigned the α-D-glucopyranosyl(1→3) stereochemistry.

^{13}C NMR assignments for Sch 20562

Most of the ^{13}C NMR chemical shifts observed for 1 were assigned by comparison with known data for the individual units in the molecule.[2,17] The assigments for each of the residues in 1 are summarized in Table 4.

Conclusion

On the basis of the data presented here, the complete structure and stereochemistry 1 was assigned to the antifungal antibiotic Sch 20562. This natural product was found to be a macrocyclic glucosidic dehydro nonapeptide lactone containing some noteworthy structural features: a high content of D-amino acids, two E-α-aminocrotonyl residues and an α-D-glucopyranosyl(1→3)-N-Me-L-allo-Thr unit in the macrocyclic lactone ring. Microbial products which are cyclic peptides containing aminocrotonic residues are not common and our literature search showed only two other natural products reported previously viz. the antifungal antibiotics stendomycin,[16] and the herbicolins A and B.[17] The structure of Sch 20562 (1) is closely related to that of herbicolin A[17] and it is probable that the producing organisms for these compounds are related taxonomically.

In summary, we describe here our studies that led to the structure elucidation of the antifungal antibiotic Sch 20562 (1). Ozonolysis of the dehydropeptide units was utilized to accomplish the selective cleavage of the peptide into three fragments which were sequenced by mass spectrometry. The stereochemistry of the amino acid units was assigned by isolation of free amino acids from the hydrolysates of the fragments. Minor chemical modifications of the fragments prior to hydrolyses were necessary to allow the selective isolation of the individual threonine units. The stereochemistry of the aminocrotonic acid units and the glucosidic linkage were assigned by NMR spectroscopy, and molecular rotation data respectively. Previously described structure elucidations of the related dehydropeptides stendomycin,[16] and the herbicolins A and B,[17] were based on mild acid hydrolyses which afforded non-selective peptide cleavages.

Experimental

General Procedures

(a) Amino acid analyses were performed on total hydrolysates (6 N HCl/110°C/18 hours) of the peptides (5 mg) and are expressed as relative ratios. (b) Permethylations were performed by methodology described previously[7] as follows: A solution of the peptide (10~20 mg) in DMSO (0.2 ml) was added with stirring at room temperature to a solution of methylsulfinyl carbanion (1~2 ml), freshly prepared from DMSO (1~2 ml) and sodium hydride (20~40 mg) at 80°C. After 5 minutes, MeI (0.1 ml) was added and the reaction was worked up after 1 hour by diluting with ice/water, acidification to pH 4 with dilute acid and extraction with CH_2Cl_2. The major product from the permethylation was isolated by TLC on silica-gel.

Sch 20562 (1)

The fermentation of *Aeromonas* sp. W-10 and the isolation of 1 from the W-10 antibiotic complex has been described.[1] Compound 1 was obtained as a white amorphous powder from MeOH: mp 170~175°C; $[\alpha]_D$ −60° (5% aq. pyridine, $c=0.4$); IR (nujol) 1653, 1730 cm^{-1}; λ_{max} (MeOH/OH$^-$) 240 nm (ε 27000), 292 nm (ε 2370); ^{1}H NMR (DMSO-d_6 100 MHz) δ 0.87 (t, 3H, $J=7$ Hz), 0.96~1.15 (ddd, 9H $J=7$ Hz), 1.25 (bs, 20H), 1.78, 1.82 (dd, 6H, $J=7$ Hz), 2.98 (s, 3H), 5.80, 5.84 (dq, 2H, $J=7$ Hz), 6.61 (d, 2H, $J=8$ Hz), 6.81 (s, 1H), 7.01 (d, 2H, $J=8$ Hz), 7.62 (s, 1H), 9.10 (s, 1H), 9.40 (s, 1H),

9.64 (s, 1H), 11.88 (s, 1H); MS (FAB) m/z 1357 (MH$^+$); Amino acid analysis: His (1), Thr (2), Glu (1), Gly (1), Tyr (1), NH$_3$ (3).

3-Ethyl-2-quinoxalinol (3) from Acid Hydrolysis of 1

A mixture of 1 (0.1 g) and 1 N HCl (10 ml) was heated at 110°C for 17 hours, cooled in ice and filtered on a celite pad. *o*-Phenylenediamine (0.1 g) was added to the clear filtrate and heated on the steam bath for 10 minutes. The crystalline product was filtered and upon recrystallization from methanol-water afforded 3 as fluffy needles: (17 mg); mp 198~200°C; ^{1}H NMR (CDCl$_3$, 60 MHz) δ 1.40 (t, 3H, $J=8$ Hz), 3.06 (q, 2H, $J=8$ Hz); identical (TLC, mp, NMR) with a reference sample.[4]

D-β-Hydroxymyristic Acid (4a) from Base Hydrolysis of 1

A solution of 1 (0.2 g) in 15% NaOH (3 ml) was heated at 110°C in a teflon pressure tube for 42 hours, cooled to room temperature, acidified with dil HCl and extracted with CH_2Cl_2. Evaporation of the extract followed by crystallization from hexanes afforded 4a as colorless crystals: (20 mg); mp 72~73°C; $[\alpha]_D$ −15.8° (CHCl$_3$, $c=0.3$). Reported[6]: mp 73~74°C; $[\alpha]_D$ −16° (CHCl$_3$, $c=2$). *Anal* Calcd for $C_{14}H_{28}O_3$: C, 68.81; H, 11.55. Found: C, 68.66; H, 11.64.

Treatment of 4a with ethereal CH_2N_2 afforded methyl D-β-hydroxymyristate (4b) as an oil that was purified by sublimation to yield a wax: mp 41°C; $[\alpha]_D$ −8° (pyridine, $c=4.3$); ^{1}H NMR (CDCl$_3$, 60 MHz) δ 0.89 (t, 3H), 1.28 (bm, 18H), 1.40 (m, 2H), 2.46 (q, 1H, $J=15$, 4 Hz), 2.51 (q, 1H, $J=15$, 3 Hz), 2.86 (bs, 1H), 3.75 (s, 3H), 4.01 (bm, 1H); MS (EI) m/z 258 (M$^+$).

Ammonolysis of 4b with methanolic ammonia afforded D-β-Hydroxymyristamide (4c) which was crystallized from MeOH - Et$_2$O as colorless needles: mp 110~111°C; $[\alpha]_D$ −4° (DMF, $c=0.65$); ^{1}H NMR (DMSO-d_6 100 MHz) δ 0.87 (bt, 3H), 2.14 (d, 2H, $J=6$ Hz), 4.55 (d, 1H, $J=4.5$ Hz), 6.78 (bs, 1H), 7.24 (bs, 1H); ^{13}C NMR (DMSO-d_6 25 MHz) δ 13.9, 22.1, 25.2, 28.8, 29.2, 31.4, 37.0, 43.3, 67.6, 173.9; MS (EI) m/z 243 (M$^+$). *Anal* Calcd for $C_{14}H_{29}O_2N$: C, 69.09; H, 12.01, N, 5.76. Found: C, 69.03; H, 12.15; N, 5.51.

1-Methyl-α-D-glucoside (5) from the Methanolysis of 1

A solution of 1 (0.4 g) in 6 N methanolic HCl (10 ml) was allowed to stand at room temperature for 20 hours, evaporated to dryness and the residue was chromatographed on silica-gel (15 g) eluting with 5% MeOH - CH_2Cl_2. The anomeric mixture of methyl glucosides

(50 mg) thus isolated was peracetylated with Ac_2O (1 ml) in pyridine (2 ml) for 24 hours at room temperature, evaporated to dryness and the product was chromatographed on silica-gel (4 g, 8% Me_2CO-hexanes) to afford the pure peracetates of the α-glucoside (47 mg) and β-glucoside (30 mg). The α-glucoside tetraacetate was dissolved in 75% methanol - ammonia (1.5 ml) and after 4 hours the solution was evaporated, the residue was dissolved in water followed by lyophilization and crystallization from $EtOH$ - Et_2O to afford 1-methyl-α-D-glucoside 5 as needles: (21 mg) mp 166°C; $[\alpha]_D$ +158° (H_2O, $c = 0.06$); ^1H NMR (DMSO-d_6-D_2O 300 MHz) δ 3.27 (s, 3H), 4.55 (d,1H, $J = 3.6$ Hz). Identical (TLC, mp, $[\alpha]_D$, NMR) with an authentic sample of 1-methyl-α-D-glucoside.

Acetaldehyde Dimedone Adduct (8) from Ozonolysis of 1

A solution of 1 (0.3 g) in MeOH (10 ml) was ozonized at −70°C followed by addition of Me_2S (0.1 ml). The solution was diluted with H_2O (10 ml) and filtered thru Celite and a solution of dimedone (0.12 g) in H_2O (1 ml) was added to the clear filtrate. The solution was heated on the steam bath and was then allowed to stand overnite in the refrigerator. The crystalline precipitate was filtered and recrystallized from MeOH-H_2O to afford 8 as colorless crystals: (55 mg) mp 141°C; ^1H NMR (CDCl$_3$, 60 MHz) δ 1.06 (s, 12H), 1.45 (d, 3H, $J = 8$ Hz), 2.25 (s, 8H), 4.10 (q, 1H, $J = 8$ Hz); identical (TLC, mp, NMR) with an authentic sample of 8.

D-Hma-E-Aca-L-Thr-D-$allo$-Thr-D-Tyr-E-Aca-D-Gln-Gly-[α-D-glucopyranosyl (1→3)-N-Me-L-$allo$-Thr]-L-His-OCH$_3$ (6)

A solution of 1 (1.0 g) in MeOH (50 ml) and Et_3N (3.0 ml) was stirred at room temperature for 24 hours and then evaporated under reduced pressure. The residue was dissolved in MeOH and diluted with Et_2O. The resulting suspension was filtered to afford 6 as a white amorphous solid: (0.95 g) mp 138 ~ 145°C; $[\alpha]_D$ −12° (pyridine, $c = 0.7$); λ_{max} (MeOH/OH⁻) 240 nm (ε 27600), 292 nm (ε 3360); IR (nujol) 1653, 1739 cm⁻¹; ^1H NMR (CDCl$_3$, 60 MHz) δ 0.80 ~ 1.15 (m, 12H), 1.83 (d, 6H, $J = 7$ Hz), 3.64 (s, 3H), 5.76 (m, 2H), 6.67 (d, 2H, $J = 8$ Hz), 7.06 (d, 2H, $J = 8$ Hz), 7.62 (s, 1H), 9.14 (s, 1H), 9.26 (s, 1H), 9.62 (s, 1H). *Anal* Calcd for $C_{64}H_{100}O_{22}N_{12}$: C, 55.32; H, 7.25, N, 12.10. Found: C, 54.83; H, 7.40; N, 11.90.

Permethylation of 6 afforded 10: HRMS(EI) (*a*) calcd for $C_{20}H_{36}O_3N$ 338.26952 (*b*) $C_{26}H_{47}O_5N_2$ 467.34849

(*c*) $C_{32}H_{58}O_7N_3$ 596.42747 (*d*) $C_{43}H_{71}O_9N_4$ 787.52210 (*e*) $C_{48}H_{78}O_{10}N_5$ 884.57486 (*f*) $C_{56}H_{92}O_{12}N_7$ 1054 (LR), found 338.27016, 467.34954, 596.42947, 787.52380, 884.57178, 1054.

Ozonolysis of 6

A stream of ozone was bubbled into a solution of 6 (1.0 g) in MeOH (60 ml) at −78°C until a blue color developed followed which, excess O_3 was removed with a stream of N_2 followed by the addition of Me_2S (2 ml). The solution was stirred at room temperature until a starch-iodide test was negative, and then Et_3N (1 ml) was added followed by evaporation under reduced pressure. The residue was chromatographed on silica-gel (80 g). Elution with 10% MeOH - CH_2Cl_2 afforded 4c as a white crystalline solid (0.17 g). Further elution with the same solvent afforded MeO-Oxalyl-L-Thr-D-$allo$-Thr-D-Tyr-NH$_2$ (15) which was crystallized from acetone as granular crystals: (0.20 g) mp 130 ~ 135°C; $[\alpha]_D$ +9.5° (MeOH, $c = 0.67$); ^1H NMR (DMSO-d_6, 100 MHz) δ 0.95 ~ 1.08 (dd, 6H, $J = 6$ Hz), 3.81 (s, 3H), 6.64 (d, 2H, $J = 8$ Hz), 7.06 (d, 2H, $J = 8$ Hz), 7.95 ~ 8.35 (m, 3H), 9.10 (s, 1H). Amino acid analysis: Thr (2), Tyr (1), NH_3 (1). *Anal* Calcd for $C_{20}H_{28}O_9N_4$: C, 51.27; H, 6.02, N, 11.96. Found: C, 51.38; H, 6.11; N, 1.16.

Elution with 20% MeOH - CH_2Cl_2 afforded MeO-Oxalyl-D-Gln-Gly-[α-D-glucopyranosyl(1→3)-N-Me-L-$allo$-Thr]-γ-N-formyl-L-Asn-OCH$_3$ (16) which was precipitated from MeOH - Et_2O as a white amorphous solid: (0.31 g) mp 128 ~ 134°C; $[\alpha]_D$ +10.4° (MeOH, $c = 0.98$); IR (nujol) 1667, 1739 cm⁻¹; ^1H NMR (DMSO-d_6-D_2O, 100 MHz) δ 0.95 ~ 1.10 (dt, 6H), 2.92 (s, 3H), 3.80 (s, 3H), 8.05 (s, 1H); Amino acid analysis Asp (1), Gly (1), Glu (1), NH_3 (2). *Anal* Calcd for $C_{27}H_{42}O_{17}N6 \cdot H_2O$: C, 43.78; H, 5.99, N, 11.35. Found: C, 43.78; H, 6.00; N, 12.17.

NH$_2$-Oxalyl-L-Thr-D-$allo$-Thr-D-Tyr-NH$_2$ (15a)

Methyl ester 15 (0.05 g) was dissolved in 15% NH_3 - MeOH (2 ml) at room temperature and after 0.5 hours was evaporated to dryness. The residue was crystallized from H_2O - Me_2CO to afford the amide 15 as colorless prisms: mp 244 ~ 245°C; $[\alpha]_D$ +7.5° (DMF, $c = 0.59$); λ_{max} (MeOH/OH⁻) 245 nm (ε 9900), 295 nm (ε 2000); IR (nujol) 1653, 1739 cm⁻¹; ^1H NMR (DMSO-d_6, 100 MHz) δ 0.98 (d, 3H, $J = 6$ Hz), 1.04 (d, 3H, $J = 6$ Hz), 6.64 (d, 2H, $J = 8$ Hz), 7.05 (d, 2H, $J = 8$ Hz), 7.84 ~ 8.60 (m, 5H), 9.08 (s, 1H). *Anal* Calcd for $C_{19}H_{27}O_8N_5$: C, 50.32; H, 6.00, N, 15.45. Found: C, 50.01; H, 6.24; N, 15.07.

Permethylation of **15a** afforded **17**: MS(EI) m/z (a) 229 (b) 358 (c) 593.

MeO-Oxalyl-L-Thr-D-*allo*-Thr-D-(O-methyl)-Tyr-NH₂(**15b**)

A solution of **15** (0.1 g) in MeOH (2 ml) was treated with excess CH_2N_2 - Et_2O. The solution was stored at 0°C for 8 hours and was then evaporated to dryness. The product was purified by chromatography on two silica gel thick-layer plates ($20 \times 20 \times 0.1$ cm) using 15%MeOH/CH_2Cl_2 as the developing solvent to afford **15b** as a white powder: ¹H NMR (DMSO-d_6, 100 MHz) δ 1.02 (d, 3H, $J=6$ Hz), 1.08 (d, 3H, $J=6$ Hz), 3.73 (s, 3H), 3.83 (s, 3H), 6.82 (d, 2H, $J=8$ Hz), 7.20 (d, 2H, $J=6$ Hz), 8.05~8.48 (m, 3H).

MeO-Oxalyl-(O-acetyl-L-Thr)-(O-acetyl-D-*allo*-Thr)-(O-acetyl-D-Tyr)-NH₂ (**15c**)

A solution of **15** (0.1 g) in pyridine (2.0 ml) and Ac_2O (1.0 ml) was stored at 15°C for 24 hours and was then evaporated under reduced pressure and azeotroped with benzene. The crude product was dissolved in CH_2Cl_2 followed by the slow addition of Et_2O to afford **15c** as a whithe amorphous solid: ¹H NMR (DMSO-d_6, 100 MHz) δ 1.08 (d, 3H, $J=6$ Hz), 1.18 (d, 3H, $J=6$ Hz), 1.94, 1.97, 2.26 (s, 9H), 3.84 (s, 3H), 7.00 (d, 2H, $J=8$ Hz), 7.28 (d, 2H, $J=6$ Hz), 8.18~8.66 (m, 3H).

MeO-Oxalyl-D-Gln-Gly-[tetra-acetyl-α-D-glucopyranosyl(1→3)-N-Me-L-*allo*-Thr]-γ-N-formyl-L-Asn-OCH₃ (**16b**)

A solution of **16** (0.05 g) in pyridine (1.0 ml) and Ac_2O (0.5 ml) was stored at 15°C for 24 hours and was then evaporated under reduced pressure. The crude product was purified by chromatography on two silica gel thick-layer plates ($20 \times 20 \times 0.1$ cm) developed with 15%MeOH/CH_2Cl_2 to afford **16b** as a white amorphous solid: ¹H NMR (CDCl₃, 100 MHz) δ 1.05 (d, 3H, $J=6$ Hz), 2.02 (s, 6H), 2.08 (s, 3H), 2.10 (s, 3H), 3.40 (s, 3H), 3.80 (s, 3H), 3.92 (s, 3H).

NH₂-Oxalyl-D-Gln-Gly-[α-D-glucopyranosyl(1→3)-N-Me-L-*allo*-Thr]-L-Asn-NH₂ (**16a**)

A solution of **16** (0.22 g) in MeOH (20 ml) was cooled in an ice-bath and saturated with NH_3. The solution was kept at 10°C for 72 hours and was then evaporated to dryness. The product was isolated by trituration with Me_2CO to afford **16a** as a white amorphous solid: (0.2 g) mp 122~132°C; $[\alpha]_D$ +11.5° (DMF, $c=0.68$); ¹H NMR (DMSO-d_6, 100 MHz) δ 1.08 (bd, 3H, $J=6$ Hz), 2.95 (s,

3H). *Anal* Calcd for $C_{24}H_{40}O_{14}N_8 \cdot H_2O$: C, 42.23; H, 6.20, N, 16.42. Found: C, 42.13; H, 6.18; N, 15.67.

Perdeuteriomethylation of **16a** afforded **18**: MS(EI) m/z (a) 285 (b) 359 (c) calcd for $C_{30}H_{22}D_{30}O_{12}N_5$ 704.5495, found 704.5493 (d) 920 (M⁺).

D-Hma-E-Aca-L-Thr-D-*allo*-Thr-D-Tyr-E-Aca-D-Gln-Gly-[α-D-glucopyranosyl (1→3)-N-Me-L-*allo*-Thr]-L-His-NHEt (**6a**)

A solution of **1** (1.0 g) in DMF (10 ml) and $EtNH_2$ (0.25 ml) was kept at 15°C for 5 days and was then evaporated under reduced pressure. The product was purified by chromatography on silica-gel (30 g), using CH_2Cl_2 - MeOH - NH_4OH - H_2O (60 : 30 : 3 : 2 v/v) as the eluting system to afford **6a** as a white amorphous solid from MeOH - Et_2O: (0.6 g) mp 150~154°C; $[\alpha]_D$ −13.2° (pyridine, $c=0.6$); λ_{max} (MeOH/OH⁻) 240 nm (ε 25700), 292 nm (ε 3380); IR (nujol) 1653 cm⁻¹; ¹H NMR (DMSO-d_6, 100 MHz) δ 0.80~1.10 (m, 15H), 1.66, 1.80 (dd, 6H), 6.36 (m, 1H), *Anal* Calcd for $C_{65}H_{103}O_{21}N_{13}$: C, 55.66; H, 7.40, N, 12.98. Found: C, 55.61; H, 7.56; N, 12.78.

MeO-Oxalyl-D-Gln-Gly-[α-D-glucopyranosyl(1→3)-N-Me-L-*allo*-Thr]-γ-N-formyl-L-Asn-NHEt (**16c**)

Ethyamide **15** (1.0 g) in MeOH (60 ml) was ozonized at −78°C as described above for **6**. The reaction product was chromatographed on silica-gel (30 g). Elution with 10% MeOH - CH_2Cl_2 afforded **4c** and **15**. Elution with 20% MeOH - CH_2Cl_2 afforded **16c** which was pre-cipitated from MeOH - Et_2O as a white amorphous solid (0.25 g): ¹H NMR (DMSO-d_6, 100 MHz) δ 1.05 (m, 6H), 2.95 (s, 3H), 3.80 (s, 3H). *Anal* Calcd for $C_{28}H_{45}O_{16}N_7 \cdot H_2O$: C, 44.62; H, 6.29, N, 13.01. Found: C, 44.86; H, 6.27; N, 13.31.

Permethylation of **16c** afforded **18a**: HRMS(EI) (a) calcd for $C_{11}H_{17}O_5N_2$ 257.11374 (b) $C_{14}H_{22}O_6N_3$ 328.15086 (c) $C_{29}H_{49}O_{13}N_4$ 661.32960 (d) $C_{39}H_{69}O_{15}N_7$ 875.48513 found 257.11384, 328.15298, 661.33034, and 875.48967 (M⁺).

MeO-Oxalyl-L-Thr-(O-tetrahydropyranyl-D-*allo*-Thr)-(O-tetrahydropyranyl D-Tyr)-NH₂ (**20**)

A solution of **1** (1.0 g) in DMF (6 ml) and dihydropyran (20 ml) containing p-TSA (0.1 g) was heated in an oil bath at 115°C for 1 hour. The clear solution was cooled to 10°C, stirred for 10 minutes with KOAc (0.2 g) and then evaporated under reduced pressure. The residue was triturated with Et_2O, the solid was filtered, washed with water and dried. The resulting per-THP **1** [IR (nujol)

1664, 1745 cm^{-1}] was dissolved in MeOH (60 ml) and ozonized at $-78°C$ as described above for 6. After the addition of Me$_2$S, the solution was stirred until a starch-iodide test was negative and then treated with Et$_3$N (2 ml). The reaction was allowed to stand at room temperature for 24 hours following which it was evaporated under reduced pressure and the product was chromatographed on silica gel (30 g). Elution with 2% MeOH - CH$_2$Cl$_2$ afforded D-β-tetrahydropyranyloxy-myristamide (4d) as a diasteroisomeric mixture which was crystallized from hexane as colorless needles: (0.16 g) mp 65~74°C; [α]$_D$ $-6.5°$ (MeOH, $c=0.85$); ^{1}H NMR (CDCl$_3$, 60 MHz) δ 0.90 (bt, 3H), 1.28 (s, 20H), 2.48 (m, 2H). *Anal* Calcd for C$_{19}$H$_{37}$O$_3$N·0.5H$_2$O: C, 67.82; H, 11.38; N, 4.16. Found: C 67.72, H, 11.10, N 4.48. Further elution with the same solvent afforded 20 which was crystallized from acetone-hexane as colorless prisms: (0.8 g) mp 110~117°C; [α]$_D$ $+21.2°$ (MeOH, $c=0.68$); *Anal* Calcd for C$_{30}$H$_{44}$O$_{11}$N$_4$·0.5H$_2$O: C, 55.80; H, 7.02, N, 8.68. Found: C, 55.69; H, 7.13; N, 8.53.

Permethylation of 20 afforded 21: MS(EI) m/z (*a*) 216 (*b*) 415 (*c*) 720 (M$^+$).

D-Tyrosine (22) from Amino Acid Unit #4

A solution of 15 (0.3 g) in 6 N HCl (10 ml) was heated at 110°C for 17 hours and was then evaporated under reduced pressure. The residue was chromatographed on silica-gel eluting with CH$_2$Cl$_2$ - MeOH - NH$_4$OH - H$_2$O (60:30:3:2 v/v). Fractions containing threonine were combined and crystallized from water - MeOH to afford a 1:1 mixture of L-threonine and D-*allo*-threonine 23 (43 mg): [α]$_D$ $-19°$ (H$_2$O, $c=0.4$); ^{1}H NMR (D$_2$O, 80 MHz) δ 1.24 (d, $J=7$ Hz), 1.32 (d, $J=7$ Hz), 3.50 (d, $J=5$ Hz), 3.78 (d, $J=4$ Hz). Fractions homogeneous in tyrosine were evaporated, the residue was dissolved in water (0.2 ml) by adding NH$_4$OH followed by acidification with AcOH and the solution was stored at 10°C overnight. The resulting crystalline product was filtered, washed with EtOH and dried to afford D-tyrosine 22 (23 mg): mp 280~285°C; [α]$_D$ $+8°$ (5 N HCl, $c=0.36$); identical (TLC, NMR, rotation) with an authentic sample of D-tyrosine.

D-*allo*-Threonine (26) from Amino Acid Unit #3

A solution of 15 (0.3 g) in 1 N HCl was heated at 70°C for 20 hours and was then evaporated under reduced pressure. The resulting ninhydrin positive 24 was dissolved in H$_2$O (15 ml), the pH of the solution was adjusted to neutrality with NaHCO$_3$ followed by the addition of NaIO$_4$ (0.318 g). After stirring for 3 hours,

ethylene glycol (0.092 g) was added and after 0.5 hour the mixture was evaporated to dryness. The residue was suspended in MeOH and filtered. The filtrate was evaporated, dissolved in H$_2$O (10 ml) and filtered thru Amberlite IR120 strongly acidic ion-exchange resin (15 ml). The eluate was evaporated to dryness to afford crude 25 (0.28 g) which was hydrolyzed in 6 N HCl (10 ml) at 110°C for 16 hours. The hydrolysate was chromatographed on silica-gel (5 ml). Elution with CH$_2$Cl$_2$ - MeOH - NH$_4$OH - H$_2$O (60:30:3:2 v/v) yielded D-tyrosine followed by the more polar fraction which after recrystallization from water-EtOH afforded D-*allo*-threonine (26) as colorless needles (24 mg): [α]$_D$ $-25°$ (5 N HCl, $c=0.35$), $-22.8°$ (H$_2$O, $c=0.47$); ^{1}H NMR (D$_2$O, 100 MHz) δ 1.24 (d, 3H, $J=7$ Hz), 3.80 (d, $J=4$ Hz); identical (TLC, NMR, rotation) with an authentic sample of D-*allo*-threonine.

L-Threonine (29) from Amino Acid Unit #2

A solution of 20 (0.2 g) in pyridine (2.0 ml) and Ac$_2$O (0.2 ml) was allowed to stand at room temperature for 24 hours and was then evaporated to dryness under reduced pressure. The residue was purified by chromatography on silica-gel (5 g, elution with 5% MeOH/CH$_2$Cl$_2$) and the product was dissolved in 70% AcOH - H$_2$O (10 ml). After 3 hours at room temperature the solution was evaporated to dryness, the residue was dissolved in acetone (5 ml) and excess Jones reagent (0.3 ml) was added. The solution was then treated with *i*-PrOH (0.2 ml), filtered thru a celite pad and the filtrate was evaporated to dryness. The residue was hydrolyzed in 6 N HCl (5 ml) at 110°C for 16 hours. The hydrolysate was chromatographed on silica gel (5 g). Elution with CH$_2$Cl$_2$ - MeOH - NH$_4$OH - H$_2$O (60:30:3:2 v/v) yielded D-tyrosine followed by the more polar fraction which after recrystallization from water - EtOH afforded L-threonine (29) as colorless needles (5 mg): [α]$_D$ $-23°$ (5 N HCl, $c=0.24$); ^{1}H NMR (D$_2$O, 100 MHz) δ 1.32 (d, 3H, $J=7$ Hz), 3.50 (d, $J=5$ Hz); identical (TLC, NMR, rotation) with an authentic sample of L-threonine.

D-Glutamic Acid (30), L-Aspartic Acid (34), and N-Methyl-L-*allo*-threonine (34) from Amino Acid Units #6, 8 and 9

A solution of 16 (0.5 g) in H$_2$O (10 ml) was stirred with NaIO$_4$ (0.6 g) at room temperature for 3 hours and was then quenched by the addition of ethylene glycol (0.1 g). After 1 hour, the pH of the solution was adjusted to neutrality with NaHCO$_3$ and the mixture was evaporated to dryness. The crude 32 obtained by extracting the

residue with MeOH was dissolved in AcOH (10 ml) and the solution was then stirred with Zn dust (2 g) at room temperature/1 hour followed by 100°C/10 minutes. The mixture was then cooled, filtered and evaporated to dryness. The resulting **33**, was hydrolyzed in 6 N HCl (20 ml) at 110°C for 24 hours and the hydrolysate was then evaporated to dryness. The residue was chromatographed on silica-gel (30 g). Elution with CH_2Cl_2-MeOH-NH_4OH-H_2O (60:30:3:2 v/v) afforded 92 mg of N-methyl-L-allo-threonine (**34**) which was crystallized from MeOH as colorless needles: mp 247～254°C; $[\alpha]_D$ +19.2° (5 N HCl, $c=0.25$), +6° (H_2O, $c=0.25$); ^1H NMR (D_2O 60 MHz) δ 1.21 (d, 3H, $J=7$ Hz), 2.76 (s, 3H), 3.62 (d, 1H, $J=4$ Hz), 4.30 (o, ^1H, $J=4, 7$ Hz), identical (TLC, NMR, rotation) with an authentic sample of N-methyl-L-allo-threonine.[13] Subsequent fractions afforded glycine. The column was then eluted with 20% NH_4OH-MeOH (200 ml) and the eluate was evaporated to dryness. The resulting residue was dissolved in H_2O (5 ml), $CuCO_3$ (200 mg) was added, the suspension was heated on a steam-bath for 0.5 hours and filtered hot. The dark blue filtrate was stored at 10°C overnite and the insoluble ASP-copper salt was isolated by filtration. The filtrate containing the soluble Glu-copper salt was treated with excess H_2S and filtered. The clear filtrate was evaporated to dryness and the residue was recrystallized from H_2O-EtOH to afford D-glutamic acid **30** as colorless crystals (15 mg): $[\alpha]_D$ $-27°$ (5 N HCl, $c=0.45$), identical (TLC, NMR, rotation) with an authentic sample of D-glutamic acid. The insoluble Asp-copper salt was suspended in water (2 ml) and stirred with H_2S at room temperature. The resulting black suspension was then filtered and the clear filtrate was evaporated to dryness. The residue was recrystallized from H_2O-EtOH to afford L-aspartic acid (**31**) as colorless crystals (25 mg): $[\alpha]_D$ +23° (5 N HCl, $c=0.32$), identical (TLC, NMR, rotation) with an authentic sample of L-aspartic acid.

Z-α-Aminocrotonic Acid Methyl Ester (37)

A solution of L-threonine (1.4 g) in AcOH (30 ml) and H_2O (3 ml) was saturated with HCl gas and after 48 hours at rt the solution was evaporated to dryness. The resulting O-acetyl-L-threonine was dissolved in NH_4OH (15 ml) at 0°C and after 48 hours at room temperature the solution was evaporated to dryness. The product was suspended in MeOH (5 ml) and treated with an excess CH_2N_2 in Et_2O, filtered and the filtrate on evaporation afforded N-acetyl-L-threonine methyl ester (0.5 g) which was dissolved in pyridine (10 ml) and treated with mesyl chloride (0.5 ml) at 0°C for 0.5 hour followed by 0.5 hour

at room temperature. The reaction mixture was then evaporated under reduced pressure, the residue was chromatographed on silica gel (15 g). Elution with 1% MeOH-CH_2Cl_2 afforded **36** as an oil (0.3 g) which was dissolved in Me_2CO (1 ml) containing Et_3N (0.1 ml) and after standing at room temperature for 24 hours the solution was evaporated and the product was purified by chromatography on silica-gel (10 g). Elution with CH_2Cl_2 afforded **39** (48 mg) followed by **37** which was crystallized from Et_2O-hexane as colorless needles: (0.15 g) mp 52～55°C; λ_{max} (MeOH) 225 nm (ε 10,200); ^1H NMR (DMSO-d_6, 100 MHz) δ 1.70 (d, 3H, $J=7$ Hz), 1.95 (s, 3H), 3.68 (s, 3H), 6.48 (q, 1H, $J=7$ Hz), 9.22 (bs, 1H). Anal Calcd for $C_7H_{11}O_3N \cdot 0.3 H_2O$: C, 51.63; H, 7.24, N, 8.41. Found: C, 51.72; H, 7.19; N, 8.62.

E-α-Aminocrotonic Acid Methyl Ester (39)

A solution of N-acetyl-L-allo-threonine methyl ester (2.0 g), prepared from L-allo-threonine using the procedure described for **37**, in pyridine (20 ml) was reacted with mesyl chloride (1.3 ml) at 0°C for 3 hours followed by addition of MeOH (0.6 ml) and evaporation under reduced pressure. The crude **38** was dissolved in Me_2CO (30 ml) containing Et_3N (3 ml) and after standing at rt for 24 hours the solution was evaporated and the product was purified by chromatography on silica gel (60 g). Elution with CH_2Cl_2 afforded **39** which was crystallized from Et_2O-hexane as colorless needles: (0.16 g) mp 48～50°C; λ_{max} (MeOH) 232 nm (ε 8600); ^1H NMR (DMSO-d_6, 100 MHz) δ 1.82 (d, 3H, $J=7$ Hz), 1.96 (s, 3H), 3.75 (s, 3H), 5.90 (q, 1H, $J=7$ Hz), 9.55 (bs, 1H). Anal Calcd for $C_7H_{11}O_3N \cdot 0.6 H_2O$: C, 50.05; H, 7.32, N, 8.34. Found: C, 49.82; H, 7.09; N, 8.17. Subsequent fractions afforded **37** (0.3 g).

References and Notes

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3) Abbreviations: Aca = α-aminocrotonic acid; Asp = Aspartic acid; Asn = Asparagine; Gln = glutamine; Glu = glutamic acid; Gly = glycine; His = histidine; Hma = β-hydroxymyristic acid; Thr = threonine; Tyr = Tyrosine

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9) A control experiment showed that ozonolysis of N-Ac-L-His followed by a) methanolysis and permethylation afforded permethylated N-Ac-Asn (m/e

10) 230) or, b) hydrolysis with 6 N HCl, afforded aspartic acid

Additionally, it was determined later in these studies that the glucosyl-N-Me-Thr unit in the peptide is prone to β-elimination to form 2 under acid hydrolysis conditions (see Scheme 6)

11) Aminolysis of the lactone 1 with EtNH₂ was found to proceed with a concomitant isomerization of the dehydropeptide units. The ¹H NMR olefinic resonances at δ 1.80 and 5.82 for 1 are shifted to δ 1.70 and 6.40 respectively, in 6a. Minor side-products resulting from conjugate addition of EtNH₂ to the dehydropeptide were also identified

12) The N-terminal methyloxalyl group of 15 and the nitrogen of the amide group of 4c are derived from an Aca unit linking these two fragments, and the same functionalities in fragments 16 and 15 are in turn derived from the other Aca unit linking these latter fragments

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