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RELATIONSHIPS BETWEEN THE STRUCTURE AND THE PHYTOTOXICITY OF THE FUNGAL TOXIN TENUAZONIC ACID

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Key Word Index—Tenuazonic acid; pyrrolidine-2,4-diones; rice; phytotoxicity; *Pyricularia oryzae*; structure–activity.

Abstract—Tenuazonic acid (3-acetyl 5-*sec*-butyl pyrrolidine-2,4-dione) is a metabolite produced by the fungal pathogen of rice *Pyricularia oryzae*. It inhibits growth of plants by interferring with protein synthesis at the ribosome level. We have synthesized analogues of tenuazonic acid with various substituents at C-3 and C-5. Substituents at C-5 other than *sec*-butyl or *n*-propyl, decrease the phytotoxicity of the analogues. But substitutions at C-3 abolish the toxicity. Thus, tenuazonic acid seems to have the optimal structure for phytotoxicity.

Tenuazonic acid induces rice leaf defence reactions (browning) of reactive varieties which are resistant to *P. oryzae*. Some of the analogues synthesized have a low level of phytotoxicity and are able to induce this leaf browning of the reactive rice varieties. Thus different structural features are required for phytotoxicity and for leaf browning.

INTRODUCTION

Tenuazonic acid (5*S*, 6*S*-3-acetyl 5-*sec*-butyl pyrrolidine-2,4-dione: TA) is a fungal metabolite with a broad toxicity spectrum. First detected as a growth inhibitor of tumour cells [1–3], TA was shown to have viricide [4], bactericide [2] and insecticide activities [5] and to be toxic to mammals [6, 7] and chicken [8]. Its toxicity to plant cells was discovered later, after it had been isolated from culture filtrates of phytopathogenic fungi (*Alternaria longipes* [9] and *Pyricularia oryzae* [10, 11]). The inhibition by TA of growth of mammal and plant cells is thought to come from the inhibition of protein synthesis [12, 13] at the ribosome level [14, 15]. Detailed studies on isolated mammal ribosomes have showed that the formation of the peptide bond is the main step inhibited by TA [16]. It has been shown that TA has a ribosome binding site, for it inhibits the binding of radioactive protein synthesis inhibitors (anisomycine and trichodermin) on the ribosome [17, 18]. Synthesis of tenuazonic acid from L-isoleucine [19, 20] allowed studies on its structure–activity relationship. When synthetic analogues were tested for their growth inhibition of tumour cells, almost all the compounds were less active than TA [3, 21–23]. The analogue with the D-isoleucine configuration was inactive on tumour cell growth [3]. From these studies a new inhibitor has been discovered [24, 25]. This inhibitor of eukaryotic cell growth is a Schiff base of an L-phenylalanine analogue of TA, which interacts with tubulin [26, 27]. TA has a low bactericidal activity. Attempts to raise its toxicity showed that TA derivates must have a long saturated chain at C-3 with a small substituent linked to C-5 [28–30]. Other modifications such as phenyl linked to C-5 or to nitrogen also raised bactericidal activity [3, 20]. Comparisons of the viricide activity of TA analogues showed that the thiosemicarbazone Schiff base of the L-valine analogue of TA has the most specific activity against viruses [31, 32]. From these results, it is clear that different modifications of the TA structure are required to improve its toxicity to bacteria or viruses, but none gives inhibitors as toxic to mammal cells as TA.

Only few of the synthetic TA analogues have been tested for their plant cell toxicity [33, 34] and from these different experiments it is not possible to deduce which are the structural features necessary for plant cell toxicity. Our interest in the comprehension of the mode of action of TA on rice cells, has led us to study the physicochemical properties of TA and its structure–activity relationships. TA is known to form metal complexes, but iron and magnesium components of its natural salt, are not able to reverse or to increase its toxicity to rice roots [35]. We have studied the influence of other metals on TA toxicity, and have synthesized one TA analogue which was unable to form metal complexes. TA analogues with different radicals linked to C-5 of the pyrrolidine-2,4-dione cycle have been synthesized. From the comparison of the biological activity of these compounds, we may identify the structural characteristics necessary for toxicity. Of these analogues, the L-valine derivative is a natural fungal metabolite produced by *Alternaria* sp. strains in contaminated grains [36]. But its toxicity to plants has never been measured. We have also synthesized analogues with different radicals linked to C-3 of the heterocyclic ring, since this part of the molecule is involved in metal complexation by TA. TA has another biological activity towards rice leaves. It induces a leaf browning of some *P. oryzae* resistant rice varieties. This browning is a barrier of pigmented cells around the toxin application point [37]. Such leaf browning is associated with rice defence reactions to *P. oryzae* [38, 39]. We have compared the toxicity and the browning induction ability of TA analogues to see

if these two activities were controlled by the same structural characteristics.

RESULTS AND DISCUSSIONS

Biological activity of tenuazonic acid and its diastereoisomers.

Tenuazonic acid is an amorphous product which is difficult to purify by chromatography on the preparative scale. We have examined the different impurities which can occur during its synthesis. First, we found that one of the synthetic TAs was contaminated by a red coloured pigment. We did not find any signal of this impurity in the ^{1}H NMR spectra of the crude product and we were able to separate it from TA by partition with pentane in which it is insoluble. We then compared the toxicity of the crude product and the decoloured TA. Both have the same toxicity to roots and the same leaf browning induction capacities. Secondly, we have found in another synthetic TA, small amounts of UV-absorbing compounds which could only be detected by anion HPLC. They were quantified by their absorption at 280 nm and represented at most 5% of TA absorbance at 280 nm. It should be noted that crystallization of TA as a copper complex did not remove these impurities. TA with or without detectable amounts of these impurities has the same toxicity to roots and the same leaf browning induction capacities. From these experiments it is clear that the impurities we have detected do not interfere with TA biological activity toward rice. We have found differences between the optical rotations of the synthetic TA, ranging from -100 to $-170°$. These differences might be the result of the epimerisation of TA during its synthesis, which gives a mixture of L-TA and D-*allo*-TA [20, 33]. In order to detect the D-*allo*-TA formed, we have synthesized the reference compounds L-*allo*-TA and D-TA to find the characteristic signals of the allo configuration of the *sec*-butyl side chain, for some differences in the NMR spectra of the TA diastereoisomers have already been reported (H-5) [33]. Comparison of high field ^{1}H NMR spectra of L-TA and L-*allo*-TA shows that three resonances are different between the two diastereoisomers (H-5, H-8 and H-9: Table 1). These signals have been used to detect and quantify the D-*allo*-TA in the different synthetic L-TAs and to check the optical purity of the L-*allo*-TA and D-TA synthesized (Table 2). The epimerisation occurs probably during the last step of the synthesis as the same non-cyclized precursor can give, after different cyclizations, TA with different optical rotations. The reaction time did not affect the epimerization. Cyclization with one equivalent of sodium methylate at 80° for 1,3,6, or 9 hr gave L-TA samples with the same percentage of D-*allo*-TA. But the use of more than one equivalent of sodium methylate, with respect to the non-cyclized TA precursor, led to significant epimerisation. Thus, stereochemically pure TA has been obtained by using 0.95 equivalent of sodium methylate for the cyclisation [40]. L-TA contaminated with D-*allo*-TA (24%) is as toxic and has the same leaf browning induction capacities as pure L-TA. Thus, D-*allo*-TA does not interfere with L-TA biological activities.

Table 1. ^{1}H NMR signals of L-TA and L-*allo*-TA

ppm	H-5(*d*)	Me-8(*t*)	Me-9(*d*)
L-TA (5*S*, 6*S*)	3.84	0.90	0.98
L-*allo*-TA (5*S*, 6*R*)	3.92	0.95	0.75

CD_3OD, 250 MHz, sodium salts.

Table 2. Optical rotations of L-TA, L-*allo*-TA and D-TA

	Contaminating* diastereoisomer	$[\alpha]_D^{22}$†
L-TA	6.5% D-*allo*-TA	−155
L-TA	24% D-*allo*-TA	−100
L-*allo*-TA	3% D-TA	−176
D-TA	12% L-*allo*-TA	+146

* Measured by ^{1}H NMR

† (Na salt; H_2O; c 0.2)

Effect of pH and of metals ions on TA toxicity to rice roots

Rice roots were treated with 0.3 mM TA, rinsed and transferred to a water solution. At this concentration growth was completely stopped, but when the roots were transferred to water, they started to grow again with a residual inhibition of 50% compared to non-treated roots. This reversibility occurs whatever the length of time of contact with TA (3–48 hr). We have also measured the influence of pH on TA toxicity to rice roots. TA solutions were buffered with MES (10 mM) at pH 5.5, 6 and 6.5 or adjusted at pH 6 without buffer. We did not find significant differences between these treatments. As we have already observed that Mg(II) and Fe(III) does not reverse nor increase TA toxicity to rice roots, we have studied the effects of other metals essential to growth [35]. Non-toxic metals [Ca(II), Fe(II), Mn(II)] were added to TA solutions (0.3 and 0.1 mM) buffered at pH 6, in 1:1 molar ratios. None of these metals was able to reverse TA root growth inhibition. The only TA-metal interaction occurs with TA-Mn(II) solutions which induce a red colouration of the root tips. This phenomenon may be explained by an oxidation of Mn(II) to Mn(III) which only occurs in the presence of TA. This hypothesis implies that the Mn(II) complex is absorbed by the roots and then oxidized in the root tips, for TA-Mn(II) solutions do not show this red colouration on standing. Toxic metals have been used at subtoxic concentrations [Cu(II): 3×10^{-6} M and Zn(II): 10^{-5} M]. They neither reversed nor increased the toxicity of TA to rice roots. Thus, TA toxicity to rice roots is reversible, independent of the pH and neither reversed nor increased by the metals essential for plant growth. These experiments confirm that the mechanism of TA phytotoxicity is not metal privation.

Effect of the configuration of the 5-sec-butyl chain on TA phytotoxicity

We have measured the toxicity to rice roots of serial dilutions of the TA diastereoisomers (Table 3). L-*allo*-TA

Table 3. Biological activities of TA diastereoisomers

	Root growth inhibition		Leaf assay (10 mM)	
	ID_{80}*	ID_{50}	BIC†	[leaf] necrosis
L-TA	0.12	0.04	+++	+++
L-*allo*-TA	0.50	0.17	+	0
D-TA	0.60	0.10	0	0

* ID_{80}: 80% inhibition dose (mM).
† BIC: Leaf browning induction capacities.

and D-TA have nearly the same ID_{80} and ID_{50} (0.5 mM and 0.16 mM). They are both less toxic than TA, the difference being statistically significant at 1% of error. L-*allo*-TA and D-TA were less able to induce leaf browning than L-TA for they only induced a slight browning at 10 mM, without necrosis. From these experiments we can conclude that changes in the configuration at C-5 or C-6 of the *sec*-butyl chain of L-TA decreases its ID_{80} to one-fifth of that of L-TA. These results are different from those obtained by Mikami [33] who finds the same level of toxicity to lettuce seedlings for L-TA and D-TA. Since D-TA is inactive against mammal cells [3], we can conclude that different structural features are involved in phytotoxicity and in mammal cell toxicity.

Comparison of the biological activities of 5-substituted analogues of TA

We have compared the toxicity to roots of TA analogues with different hydrophobic or polar side chains linked to C-5 of the pyrrolidine-2,4-dione cycle. These side chains have been chosen to point out the simplest structure needed for toxicity. The results are given in Table 4. Analogues with side chains shorter or longer than that of TA had a low toxicity. They were 10 times less toxic than TA (ID_{80}). The analogues with the shortest (**4**, one carbon long) or the longest (**13**, four carbons long) side chain were the least toxic of all. Analogues with a three carbons linear side-chain (substituted or not) were the most toxic, but still they were significantly less toxic than TA. Among them, the analogue with a 5-*n*-propyl chain was the most toxic to roots and induces a leaf browning similar to that of TA. Thus, the phytotoxicity of 3-acetyl pyrrolidine-2,4-diones varies with the length of the side chain linked to C-5 of the cycle. This length must be of three linear carbons. Substitutions on this side chain other than that of TA (5*S*, 6*S*, *sec*-butyl) decrease the phytotoxicity compared to TA. This suggests that the side chain of TA fits into a 'receptor site' which recognizes the 5*S*, 6*S*, *sec*-butyl configuration, and that this interaction is necessary for phytotoxicity. Modifications of the *sec*-butyl chain might lower the affinity of the analogues for this 'site' and thus decrease their toxicity compared to TA.

Comparison of the biological activities of 3-substituted TA analogues

We have compared the biological activities of 5-*sec*-butyl pyrrolidine-2,4-diones with different substituents at C-3. The 3-acetyl group is conjugated with two other carbonyl groups. This conjugation is necessary for ionization and it is responsible for the acid and metal complexing properties of TA. The oxime (**15**) and the Schiff bases derived from TA (**16**, **17**) might have metal complexation properties different from those of TA. We have also synthesized an analogue without metal complexing properties (**18**, H_2-3). Some of the 3-substituted analogues tested (**14**, **15**, **16**, **18**) had a low toxicity, the least toxic being analogue **18** (H_2-3) (Table 5). Analogues which were able to complex iron, as shown by their ability to form red coloured (**14**) or purple coloured (**15**, **16**) complexes with $FeCl_3$, were much less phytotoxic than TA. From these results it might be concluded that metal complexation of not essential for phytotoxicity, though these analogues might have a lower affinity for iron than TA. Other changes introduced by the formation of an oxime or a Schiff base might be responsible for the decrease in toxicity. First, changes in the equilibrium constants between the *Z* and *E* forms are produced by

Table 4. Biological activities of 5-substituted TA analogues

Substituent (analogue number)	Root growth inhibition		Leaf assay (10 mM)	
	ID_{80}*	ID_{50}	BIC†	necrosis
5*S*-Methyl (**4**)	4.20	0.90	0	0
5*S*, Ethyl (**5**)	1.50	0.38	0	0
5*S*-*iso*-Propyl (**6**)	1.30	0.37	++	0
5*S*-*n*-Propyl (**7**)	0.36	0.10	++	+
5*S*, 6*S*-*sec*-Butyl (L-TA, **1**)	0.12	0.04	+++	++
5*S*-*iso*-Butyl (**8**)	0.97	0.28	++	0
5*R*-*iso*-Butyl (**9**)	0.36	0.13	0	+
5*S*-*n*-Butyl (**10**)	1.80	0.60	+	0
5*S*-Benzyl (**11**)	3.40	1.50	+	0
5*S*-2-Carboxy-ethyl (**12**)	3.30	1.80	+	0
5*S*-4-Amino-butyl (**13**)	10 <	3.00	0	0

* ID_{80}: 80% inhibition does (mM).
† BIC: Leaf browning induction capacities.

Table 5. Biological activities of 3-substituted TA analogues

Substituent (analogue number)	Root growth inhibition		Leaf assay (10 mM)	
	ID_{80}*	ID_{50}	BIC†	necrosis
Acetyl (TA:**1**)	0.12	0.04	+++	++
(Carboxy-methyl) (**14**)	10 <	10.0	0	0
2-(Hydroxy-imino)-ethyl (**15**)	6.00	2.00	+	0
2-(Methyl-imino)-ethyl (**16**)	4.00	1.00	0	+
2-(Thiosemicarbazone-imino)-ethyl (**17**)	0.50	0.12	0	++
3-Dihydro (**18**)	10 <	5.00	+‡	0

* ID_{80}: 80% inhibition dose (mM).
† BIC: Leaf browning induction capacities.
‡ Non-specific browning induced on both TA-reactive (IRAT13) and TA-non-reactive (Maratelli) rice varieties.

these 3-substitutions, as shown by the intensities of their ^1H NMR signals (H-5 and H-11) which correspond to ratios different from that of TA. Secondly, these analogues might have ionization properties different from that of TA. Thus, it is difficult to understand why these analogues are less phytotoxic. Therefore, we can conclude that the acetyl group linked to the C-3 of the cycle is essential to phytotoxicity as its modifications lead to less toxic analogues.

Comparison between the toxicity and the leaf browning induction capacity of TA analogues

Rice leaf browning is a mechanism of defence against various agressions (fungi, bacteria, chemicals). It can be induced by several inhibitors structurally unrelated to TA, such as protein synthesis inhibitors (anisomycine, cycloheximide), sulphydryl reagents (iodoacetic acid [38]). But the browning induced by these chemicals is not as specific as that of TA for it is induced on all rice varieties. TA (10 μl, 1 mM) induces a rapid (48 hr) and intense leaf browning only on some rice varieties resistant to *P. oryzae* [37]. The response of other varieties is weaker and occurs later (> 4 days). When applied at higher concentrations (10 μl, 10 mM), TA also induces a leaf necrosis on all rice varieties as well as the browning of the edge of the dead area on reactive varieties. Thus, we have tested the leaf toxicity (necrosis) and the specific browning induction capacities of all the TA analogues synthesized, by treating rice leaves with drops of a 10 mM solution of each analogue. The majority of the analogues were not toxic for the leaves, and some did not induce leaf browning (Table 6). A large number were able to induce a weak browning (+) which corresponded to a small brown spot located at the application point of the drops on the leaves. Three analogues were able to induce a clear browning (++), but only one of them induced the same symptoms as TA. The analogue derived from L-valine (**6**) induced a brown ring at 0.5 to 1 cm from the application point without a necrotic area. The analogue derived from L-leucine (**8**) induced a large brown area (2–4 mm^2) without necrosis. The analogue derived from L-norvaline (**7**) induced a symptom which was similar to that of TA, with a central necrotic zone surrounded by an intense brown ring, the difference being a smaller necrotic area than TA. Leaf browning can be induced by most of the TA analogues, whatever their phytotoxicity level (leaf necrosis, ID_{80}). Some of the analogues with a lower toxicity than TA (ID_{80}: 1 mM) are able to induce an intense leaf browning (**6**, **8**). In contast one of the most toxic analogues (**9**, ID_{80}: 0.3 mM) does not induce leaf browning. Thus, this reaction is not a response of the leaf to chemical agression, but seems to involve other mechanisms. The structural characteristics needed for browning induction are less precise than those needed for root growth inhibition. The side chain linked to C-5 should be made of 3 or more carbons, with a length of 2–4 carbons. Substitution of the methyl terminal group by a polar group does not change the browning induction capacities of the compounds. The only structural requirement appears to be an *S* configuration at C-5, for the 5*R* analogues (D-TA and **9**), unlike their 5*S* isomers, are not able to induce leaf browning. For the analogues substituted at the C-3, only one of the Schiff bases (**15**) is able to induce a weak browning. All the analogues having a 3-acetyl group and a 5-substituted group close to the *sec*-butyl structure (*iso*-propyl, *n*-propyl, isobutyl) induce an intense leaf browning.

Table 6. Distribution of the TA analogues for their leaf browning induction capacities and their phytotoxicity (leaf necrosis)

	Leaf browning induction (10 mM)			
Leaf necrosis (10 mM)	None (O)	Weak (+)	Intermediate (++)	Intense (+++)
None (O)	4*	6	2	0
Weak (+)	1	0	1	0
Intense (++)	2	0	0	TA

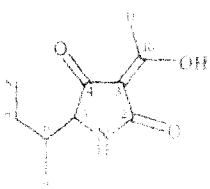
*Number of analogues in each class. **18** unclassified.

CONCLUSIONS

Modifications of tenuazonic acid at C-5 and C-3 of its pyrrolidine-2,4-dione cycle decreased the toxicities of the compounds to rice roots. The analogues which are the most toxic have a 3-acetyl and a 5-*sec*-butyl (5*S*, 6*S*: TA; 5*S*, 6*R*: L-*allo*-TA; 5*R*, 6*R*: D-TA) or a 5*S*-*n*-propyl group. Analogues with substituents in C-5 having more or less than 3 linear carbons are less toxic to rice roots than TA. Modifications of the 3-acetyl group lead to non-phytotoxic analogues. Thus, TA seems to have an optimal structure for toxicity to rice roots. Its naturally produced analogue derived from valine is only weakly phytotoxic. Leaf browning induction by TA seems to involve structural characteristics that are different from those needed for toxicity to roots. We have identified analogues with a low root toxicity which are able to induce a leaf browning as specific as that of TA. These analogues may be used to induce the defence mechanisms of the rice leaf.

EXPERIMENTAL

General procedures. We have used a carbon numbering system which is different to that of Nolte [41]. This numbering takes into account that the two asymmetric carbons of TA are linked, and allows that their numbers follow each other (Fig. 1). High field ^1H or ^{13}C NMR: 250 or 62.5 MHz. Other ^1H NMR: 90 MHz; TMS was used as an int. standard in deuterated solvents. MS: Volatile samples were introduced by GC (capillary column OV-1) coupled to the spectrometer with electron impact (EI) (GC–MS, 70 eV) or chemical ionization (CI) (GC–CI MS, NH_3, 90 eV). Other samples were submitted to desorption–chemical ionization mass



Tenuazonic acid (Z form)

Fig. 1

spectrometry (DCI-MS, NH_3, 90 eV) [42]. Specific optical rotations were measured (1 dm cell) in H_2O or in MeOH at two concentrations: (*c*1 and *c*0.2). Elementary analysis were performed by the microanalysis service of the CNRS (Gif, France). HPLC: Silica based anion exchanger prepacked analytical columns were obtained from Chrompack (ionosphere 5 or 10 μm, 3 × 150 mm or 4.6 × 250 mm). Isocratic chromatography was performed with different H_2O–MeCN mixtures (5–15 % MeCN) buffered at pH 6.5 with 0.01 M phosphate and 0.001 M EDTA. Detection 280 or 254 nm.

Synthesis of 3-acetyl tetramic acids derived from apolar aminoacids. A modified protocol of Lacey [19] and Harris [20] was used to improve the yield. Esterification of the amino acids (Fluka, Bachem) was performed in MeOH with $SOCl_2$ at 40° [43]. The quantities given in the text correspond to the synthesis of TA from L-isoleucine methyl ester. The aminoester hydrochloride (2.2 g, 12 mM) was neutralized with Et_3N (1.7 ml, 12 mM). The cooled soln (−4°) was treated dropwise with one equivalent of freshly distilled diketene (0.85 ml, 12 mM) and kept in ice for 1 hr with stirring. Then H_2O was added, the product extracted by Et_2O and chromatographed on a silica gel column with cyclohexane–EtOAc (1 : 1) to afford a colourless gum (yield: 76 %, 2.12 g, 9.2 mM). This gum (2 g, 8.7 mM) was dissolved in dry MeOH (4 ml) and treated dropwise with 0.95 equivalent of freshly prepared MeONa (0.19 g Na in 2.5 ml MeOH, 8.3 mM). This soln was heated at 80° for 1–4 hr at reflux. At the end of the reaction the MeOH was evapd and H_2O was added. This soln was extracted with EtOAc. The remaining H_2O phase was acidified to pH 2 and extracted with EtOAc or Et_2O to afford a gum (yield 87 %; 1.5 g, 7.6 mM). This gum was dissolved in pentane when coloured since the contaminating pigments were not soluble in that solvent. The gum was sometimes crystallized depending on the amino acid from which it was derived. Tenuazonic acid was not crystallizable. The overall yield was 65 % for tenuazonic acid and may be greater with other aminoacids. These 3-acetyl tetramic acids were neutralized by a cation exchanger (Dowex 50, Na^+) in H_2O–MeOH mixtures and lyophilized to give amorphous white sodium salts. These salts were stored at −20° in a dry atmosphere. Purity of the sodium salt was controlled by anion HPLC with UV detection at 280 nm, by elemental analysis and by high field ^1H NMR in CD_3OD. The characteristics of the 3-acetyl tetramic acids are given below for their acidic form, unless when stated (sodium salt).

Tenuazonic acid: (5S, 6S)-3-*acetyl* 5-sec-*butyl pyrrolidine*-2,4-*dione* (**1**). $[\alpha]_D^{22}$ −157° (*c*1 and 0.2; H_2O;), $[\alpha]_{546}^{22}$ −190° (*c*1 and 0.2; H_2O; Na salt), lit. [20] $[\alpha]_{546}^{22}$ −95 to −165° (Na salt; MeOH; *c* 2); $[\alpha]_D^{22}$ −120° (MeOH; *c* 0.2), lit. [33] −123° (MeOH; *c*2); UV $\lambda_{max}^{H_2O}$ nm (log ε) Na salt: 240 (4.06), 280 (4.16); ^1H NMR (CD_3OD, Na salt): δ0.9 (3H, *t*, *J* = 7 Hz, H-8), 0.98 (3H, *d*, *J* = 7 Hz, H-9), 1.25 (2H, *m*, H-7), 1.9 (H, *m*, H-6), 2.45 (3H, *s*, H-11), 3.84 (H, *d*, *J* = 3 Hz, H-5); ^1H NMR ($CDCl_3$): δ0.9 (3-H, *t*, *J* = 8 Hz, H-8), 1.0 (3H, *d*, *J* = 7 Hz, H-9), 1.25 (2H, *m*, H-7), 1.96 (H, *m*, H-6), 2.45 and 2.5 (3H, *s*, H-11, 75–25 %, *Z*-*E*, lit. [41]), 3.8 and 3.92 (H, *d*, H-5, 75–25 %, *Z*-*E*), 7.7 (H, NH); ^{13}C NMR (62.5 MHz, D_2O, Na salt): δ12.20 (*q*, C-8), 16.75 (*q*, C-9), 23.10 (*t*, C-7), 28.25 (*q*, C-11), 37.35 (*d*, C-6), 65.7 (*d*, C-5), 104.60 (*s*, C-3), 178.80 (*s*, C-2), 196.20 (*s*, C-10), 199.80 (*s*, C-4); The ^{13}C chemical shifts were attributed by reference to those described by Nolte [41] for the acidic form of TA in MeOH. Nickel forms a paramagnetic complex with TA [35]. Thus, addition of nickel (2.5 %) to the TA soln allowed a broadening of the signals at δ28.25 and 65.7. This confirms the assignment of the signal at δ28.25 to C-11 and at 65.7 to C-5, for these carbons are those which are the closest to the metal. GC-CIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 198 $[M+H]^+$ (100), 141 (40); DCI-MS, Na salt: *m*/*z* (rel. int.): 220 $[M+H]^+$ (100), 164 (30).

L-allo-*TA:* (5S, 6R)-3-*acetyl* 5-sec-*butyl pyrrolidine*-2,4-*dione* (**2**). $[\alpha]_D^{22}$ −176° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.06), 280 nm (4.14); ^1H NMR (CD_3OD, Na salt): δ0.75 (3H, *d*, *J* = 7 Hz, H-9), 0.95 (3H, *t*, *J* = 7 Hz, H-8); 1.3 (2H, *m*, H-7), 1.9 (H, *m*, H-6), 2.43 (3H, *s*, H-11), 3.92 (H, *d*, *J* = 3 Hz, H-5); DCI-MS (NH_3) 90 eV, *m*/*z* (rel. int.): 198 $[M+H]^+$ (100 %), 141 (30); (Found: C, 51.82; H, 6.58; N, 6.17. $C_{10}H_{14}N\,O_3\,Na.H_2O$ requires: C, 50.6; H, 6.7; N, 5.9 %).

D-*TA:* (5R, 6R)-3-*acetyl* 5-sec-*butyl pyrrolidine*-2,4-*dione* (**3**). $[\alpha]_D^{22}$ +146° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.03), 280 (4.12); ^1H NMR (CD_3OD): δ0.9 (3H, *t*, *J* = 7 Hz, H-8), 1.1 (3H, *d*, *J* = 7 Hz, H-9), 1.47 (2H, *m*, H-7), 1.9 (H, *m*, H-6), 2.45 (3H, *s*, H-11), 3.85 (H, *d*, *J* = 3 Hz, H-5).

(5S)-3-*Acetyl* 5-*methyl pyrrolidine*-2,4-*dione* (**4**). Mp 115°, lit. [19, 29] 115–118°; $[\alpha]_D^{22}$ −12° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.04), 280 (4.15); ^1H NMR ($CDCl_3$): δ1.38 (3H, *d*, *J* = 7 Hz, H-6); 2.45 and 2.55 (3H, *s*, H-8, 75–25 %, *Z*-*E*) 3, 9 and 4.1 (H, *qt*, *J* = Hz, H-5, 75–25 %, *Z*-*E*); 6.7 (NH); GC–EIMS 70 eV, *m*/*z* (rel. int.): 155 $[M]^+$ (89), 84 (48).

(5S)-3-*Acetyl* 5-*ethyl pyrrolidine*-2,4-*dione* (**5**). Mp 85°; $[\alpha]_D^{22°}$ −141° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.08), 280 (4.16; ^1H NMR ($CDCl_3$): δ0.95 (3H, *t*, *J* = 8 Hz, H-7), 1.75 (2H, *m*, H-6), 2.45 and 2.5 (3-H, *s*, H-9, 85–15 %, *Z*-*E*), 3.8 and 4.0 (H, *t*, *J* = 6 Hz, H-5, 85–15 %, *Z*-*E*), 7.3 (NH). DCIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 170 $[M+H]^+$ (100), 141 (20); (Found: C, 56.05; H, 6.45; N, 8.05; Calc. for $C_8H_{11}NO_3$: C, 56.04; H, 6.5; N, 8.2 %).

(5S)-3-*Acetyl* 5-n-*propyl pyrrolidine*-2,4-*dione* (**6**). Mp 87°; $[\alpha]_D^{22}$ −129 (Na salt: H_2O; *c* 0.2): UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.06), 280 (4.16); ^1H NMR ($CDCl_3$): δ0.1 (3H, *t*, *J* = 7 Hz, H-8), 1.45 (2H, *m*, H-7), 1.6 and 1.75 (2H, *m*, C-6), 2.45 and 2.5 (3H, *s*, H-10, 75–25 %, *Z*-*E*), 3.85 and 4.0 (H, *dd*, $J_{56\alpha}$ = 4Hz, $J_{56\beta}$ = 12Hz, H-5, 75–25 %, *Z*-*E*); GC-CIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 184 $[M+H]^+$ (100 %), 141 (27); (Found: C, 59.1; H, 7.14; N, 7.85. Calc. for $C_9H_{13}NO_3$: C, 59; H, 7.1; N, 7.65 %).

(5S)-3-*Acetyl* 5-iso-*propyl pyrrolidine*-2,4-*dione* (**7**). $[\alpha]_D^{22}$ −160° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.04), 280 (4.14); ^1H NMR (CDOD): δ0.8 (3H, *d*, *J* = 8 Hz, H-8), 1.0 (3H, *d*, *J* = 8 Hz, H-7), 2.15 (H, *m*, H-6), 2.5 (H-3, *s*, H-10), 3.8 (H, *d*, *J* = 4 Hz, H-5); GC-EIMS 70 eV, *m*/*z* (rel. int.): 183 $[M]^+$ (18), 141 (100).

(5S)-3-*Acetyl* 5-*isobutyl pyrrolidine*-2,4-*dione* (**8**). Mp 105°, lit. [31] 114°; $[\alpha]_D^{22}$ −50° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.05), 280 (4.15; ^1H NMR ($CDCl_3$): δ1.0 (6H, *d*, *J* = 8 Hz, H-8, H-9), 1.45 (H, *m*, H-7), 1.75 (2H, *m*, H-6), 2.45 and 2.5 (3H, *s*, H-11 75–25 %, *Z*-*E*), 3.85 and 4 (H, *dd*, $J_{56\alpha}$ = 4 Hz, $J_{56\beta}$ = 8 Hz, H-5, 75–25 %, *Z*-*E*); GC-CIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 198 $[M+H]^+$ (100), 141 (5); (Found: C, 60.95; H, 7.85; N, 7.25. $C_{10}H_{15}NO_3$ requires: C, 60.9; H, 7.61; N, 7.1 %).

(5R)-3-*Acetyl* 5-iso*butyl pyrrolidine*-2,4-*dione* (**9**). Mp 138°. $[\alpha]_D^{22}$ +134° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 240 (4.01); 280 (4.13); ^1H NMR (CD_3OD): δ0.97 (3H, *d*, *J* = 9 Hz, H-8, H-9), 1.43 (H, *m*, H-7), 1.72 (2H, *m*, H-6), 2.43 (3H, *s*, H-11), 3.95 (H, *dd*, $J_{56\alpha}$ = 4.5 Hz, $J_{56\beta}$ = 9 Hz, H-5); GC-CIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 198 $[M+H]^+$ (100), 141 (18); (Found: C, 60.8; H, 7.85; N, 6.94. $C_{10}H_{15}NO_3$ requires: C, 60.9; H, 7.61; N, 7.1 %).

(5S)-3-*Acetyl* 5-n-*butyl pyrrolidine*-2,4-*dione* (**10**). Mp 85°, lit. [44] 88°–89°; $[\alpha]_D^{22}$ −128° (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log ε), Na salt: 239 (4.03), 278 (4.16); ^1H NMR ($CDCl_3$): δ0.9 (3H, *t*, *J* = 6 Hz, H-9), 1.2–1.8 (6H, *m*, H-6, H-7, H-8), 2.45 and 2.5 (3H, *s*, H-11, 75–25 %, *Z*-*E*), 3.8 *and* 4.0 (*H*, *t*, *J* = 4 Hz, 75–25 %, *Z*-*E*); DCIMS (NH_3) 90 eV, *m*/*z* (rel. int.): 198 $[M+H]^+$ (100), 154 (5), 141 (30); (Found: C, 60.66; H, 7.65; N, 7.55. $C_{10}H_{15}NO_3$ requires: C, 60.9; H, 7.67; N, 7.1 %).

(5S)-3-*Acetyl* 5-*benzyl pyrrolidine*-2,4-*dione* (**11**). Mp 149°, lit.

[29, 31, 45] 134–155°; $[\alpha]_D^{22} - 108°$ (Na salt; H_2O; *c* 0.2), lit. [45] $-210°$ (EtOH; *c*1); UV $\lambda_{max}^{H_2O}$ nm (log *ε*), Na salt: 240 (4.07), 280 (4.17); ^1H NMR ($CDCl_3$): δ 2.45 (3H, *s*, H-14), 2.70 (H, *dd*, *J* = 8, 13 Hz, H-6), 3.3 (H, *dd*, *J* = 4, 13 Hz, H-6), 4.0 (H, *dd*, $J_{5\alpha,6}$ = 4 Hz, $J_{5\alpha,6\beta}$ = 8 Hz, H-5), 6.7 (NH), 7.2 (5H, *m*, phenyl group); DCIMS (NH_3) 90 eV, *m/z* (rel. int.): 232 $[M+H]^+$ (100), 189 (10), 140 (63); (Found: C, 67.4; H, 5.51; N, 6.17. $C_{13}H_{13}NO_3$ requires: C, 67.6; H, 5.6; N, 6.03%).

Synthesis of 3-acetyl tetramic acids derived from lysine and glutamic acid. (5S)-3-*Acetyl* 5-(2-*carboxy-ethyl*) *pyrrolidine*-2,4-*dione* (**12**). The methyl ester of the 5-*tert*-butyl ester of glutamic acid (Bachem: 0.8 g, 4 mM) was treated with diketene (0.6 ml, 8 mM) in MeOH (3 ml) at $-4°$ for 1 hr to give the acetoacetamide in 88% yield (1 g, 3.5 mM). The acetoacetamide (0.84 g, 3 mM) was dissolved in dry tertiobutanol (6 ml) and treated with one equivalent of potassium *tert*-butoxide (0.28 g, 3 mM) for 5 hr at 80°. The reaction was stopped by addition of H_2O. After acidification to pH 3.59 the H_2O phase was extracted with EtOAc. The gum obtained was chromatographed on a silica gel column with $CHCl_3$–MeOH–AcOH (89:10:1) as eluent. The purified product (0.45 g, 1.8 mM) was crystallized from MeOH (mp > 250°; yield 60%). ^1H NMR and GC–MS spectra were in agreement with the proposed structure. The tertiobutyl protecting group was removed by treatment of the ester (0.12 g, 0.5 mM) with trifluoroacetic acid (0.5 ml, 6 mM) at 35° for 5 min. The gum obtained was neutralized with $NaHCO_3$ and chromatographed on an anion exchange column (Dowex 200) eluted with 0.5 M HOAc. The product obtained was red (λ_{max} = 440 nm) and was treated with a metal exchange resin (IRC 718, Na^+) to remove the trace of iron. The final product was a slightly coloured gum. ^1H and ^{13}C NMR spectra were in agreement with the proposed structure. Mass spectrum (DCI) could not be obtained for this compound since it gave only decomposition products. $[\alpha]_D^{22} - 15°$ (H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm: 240, 280; ^1H NMR (D_2O): δ 1.8–2.2 (4H, *m*, H-6, H-7), 2.35 (3H, *s*, H-10), 3.8 (H, *m*, H-5); ^{13}C NMR (D_2O): δ 28.41 (C-10), 29.07 (C-6), 33 (C-7), 59.97 (C-5), 103.83 (C-3), 178.7 (C-2), 183.7 (C-8), 196.9 (C-9), 199.95 (C-4). The assignments of the ^{13}C NMR signals were made in agreement with the reported TA ^{13}C NMR spectra [41] and the spectra of the sodium salt of glutamic acid in D_2O.

(5S)-3-*Acetyl* 5-(4-*amino-butyl*) *pyrrolidine*-2,4-*dione* (**13**). The methyl ester of *N*-*ε*-CBZ-lysine hydrochloride (Bachem: 5.6 g, 19 mM) was treated with diketene (3.5 ml, 50 mM) in MeOH (30 ml) at $-4°$ for 1 hr to obtain its acetoacetamide, which was crystallized from Et_2O (mp 74°, yield 90%: 5.8 g, 17 mM). Then, the acetoacetamide (2 g, 5.8 mM) was cyclized with MeONa (0.13 g Na in 3 ml MeOH, 5.6 mM) in MeOH (10 ml) at 80° for 5 hr. The reaction was stopped by addition of H_2O. After acidification and extraction with EtOAc, the product crystallized (mp 104°, yield 90%: 1.8 g, 5.2 mM). ^1H NMR and DCIMS spectra were in agreement with the proposed structure. Then, the *N*-*ε*-CBZ pyrrolidine-2,4-dione (1.8 g, 5.2 mM) was dissolved in 95° EtOH (40 ml) and the soln was stirred under H_2 in the presence of a Pd/active charcoal (5%) catalyst (2 g) for 45 min to remove the CBZ protecting group. The reaction time must not exceed 1 hr to avoid the reduction of the carbonyls of the pyrrolidine-2,4-dione cycle [45]. The resulting product was removed from the charcoal by elution with 95° EtOH–NH_3 mixtures and treated with a cation exchanger (Dowex 50, Na^+). The water soluble compound obtained (yield 36%: 0.4 g, 1.9 mM) contained only small amounts of sodium ion. This molecule was presumed to be the amphoteric form of the amino acid. ^1H and ^{13}C NMR spectra were in full agreement with the proposed structure. Mass spectrum (DCI) could not be obtained with this compound as it gave only decomposition products. $[\alpha]_D^{22} - 110°$ (Na salt; H_2O; *c* 0.2); UV $\lambda_{max}^{H_2O}$ nm (log *ε*), Na salt: 240 (4.0), 278 (4.10); ^1H NMR (CD_3OD): δ 1.4–1.9 (6H, *m*, H-6, H-7, H-8), 2.4 (3H, *s*, H-11), 3.1 (2H, *t*, *J* = 8 Hz, H-9), 4.0 (H, *t*, *J* = 4 Hz, H-5); ^{13}C NMR (D_2O): δ 19.72 (C-7), 26.24 (C-8), 26.99 (C-11), 29.87 (C-6), 38.8 (C-9), 58.7 (C-5), 102.6 (C-3), 177.4 (C-2), 196.9 (C-10), 198.4 (C-4); (Found: C, 46.9; H, 6.5; N, 11.4. $C_{11}H_{18}N_2O_4$, 2.5 H_2O requires: C, 46.6; H, 8.1; N, 10.9%).

Synthesis of 3-carboxymethyl 5-*sec-butyl pyrrolidine*-2,4-*dione and of* 5-*sec-butyl pyrrolidine*-2,4-*dione*. (5S, 6S)-3-*Carboxymethyl* 5-*sec-butyl pyrrolidine*-2,4-*dione* (**14**). We followed the synthetic scheme used by Jones with valine as starting material [46]. Dicyclohexylcarbodiimide in excess (2.44 g, 11.8 mM) and methyl-L-isoleucine ester (1.43 g, 10 mM)) were added successively to a cooled soln of ethyl hydrogen malonate (1.32 g, 10 mM) in CH_2Cl_2 (2 ml). The soln was refluxed with stirring for 20 h. The filtrate was chromatographed on a silica gel column (hexane–EtOAc 1:1) to give a colourless gum (yield 33%: 1.6 g, 5.23 mM) with the following characteristics: *N*-(ethoxycarbonyl-methylene-carbonyl)-L-isoleucine methyl ester: $[\alpha]_D^{22} - 2°$ (*c*1; MeOH); ^1H NMR ($CDCl_3$): δ 0.95 (6H, *m*), 1.3 (3H, *t*, *J* = 7 Hz), 1.35 (2H, *m*), 1.9 (H, *m*), 3.35 (2H, *s*), 3.73 (3H, *s*), 4.22 (2H, *q*, *J* = 7 Hz), 4.6 (H, *dd*, *J* = 5 Hz, 9 Hz), 7.6 (NH).

The above compound (1.43 g, 5.56 mM) was dissolved in dry MeOH (2.2 ml) and cyclized with MeONa (0.95 equivalent: 0.12 g Na in 1.8 ml MeOH) at 80° for 2 hr with stirring. The precipitate obtained was filtered and acidified to pH 2 with 3M HCl. The aq. phase was extracted with $CHCl_3$ to give a crude product which contained the two epimers of the expected product as measured by high field ^1H NMR (1–90% D-*allo* 10%). Crystallization in hexane–EtOAc (1:1) gave the pure L diastereoisomer (5S, 6S) with 68% overall yield (1.08 g, 3.76 mM). Mp 104°; $[\alpha]_D^{22} - 49°$ (MeOH; *c*1); $[\alpha]_D^{22} - 112°$ (Na salt; H_2O; *c*1); UV $\lambda_{max}^{H_2O}$ nm (log *ε*), Na salt: 220 (4.21), 260 (4.06); ^1H NMR (CD_3OD): δ 0.92 (3H, *t*, *J* = 8 Hz, H-8), 1.0 (3H, *d*, *J* = 7 Hz, H-9), 1.3 (2H, *m*, H-7), 1.95 (H, *m*, H-6), 3.8 (3H, *s*, H-11), 4.1 (H, *d*, *J* = 3.5 Hz, H-5); the D-*allo* diastereoisomeric form (5R, 6S) of this compound had the same ^1H chemical shifts except for H-9, δ 0.77 (*d*, *J* = 7 Hz) and H-5, δ 4.17 (*d*, *J* = 3 Hz); DCI-MS (NH_3) 90 eV, *m/z* (rel. int.): 214 $[M+H]^+$ (100), 199 (30), 157 $[M+H-butyl]^+$ (53), 142 (23); (Found: C, 56.04; H, 7.23; O, 30.67; N, 6.17. $C_{11}H_{17}NO_4$ requires: C, 56.34; H, 7.04; O, 30.04; N, 6.57%).

(5S, 6S)-5-sec-*Butyl pyrrolidine*-2,4-*dione* (**18**). The 3-carboxymethyl tetramic acid was decarboxylated according to [47–49]. The 3-carboxymethyl 5-*sec*-butyl pyrrolidine-2,4-dione (190 mg, 0.9 mM) was dissolved in nitromethane (3 ml), 2 equivalents of distilled H_2O (33 *μ*l, 1.8 mM) added and the soln heated at reflux for 1 hr. After evapn of the solvent, the solid obtained was crystallized from hexane–EtOAc (1:1) to give white crystals with 65% yield (91 mg, 0.58 mM). Mp 115°, lit. [20] 112°; $[\alpha]_D^{22} + 40°$ (MeOH; *c*1); UV $\lambda_{max}^{H_2O}$ nm (log *ε*), pH 6: 260 (3.11); ^1H NMR ($CDCl_3$): δ 0.9 (3H, *t*, *J* = 7 Hz, H-8), 1.03 (3H, *d*, *J* = 7 Hz, H-9), 1.35 (2H, *m*, H-7), 1.9 (H, *m*, H-6), 3.0 (2H, *s*, H-3), 3.93 (H, *d*, *J* = 4 Hz, H-5); (Found: C, 61.49; H, 8.29; N, 8.99; O, 21.02. $C_8H_{13}NO_2$ requires: C, 61.93; H, 8.38; N, 9.03; O, 20.64%).

Synthesis of tenuazonic acid oxime and Schiff bases. These derivatives were prepared according to reported procedures [19, 21, 31]. (5S, 6S)-3-[2-(*Hydroxy-imino*)-*ethyl*]-5-*sec-butyl pyrrolidine*-2,4-*dione* (**15**). L-TA (0.4 g, 2 mM) was refluxed for 5 min with three equivalents of hydroxylamine chlorhydrate (0.42 g, 6 mM) and three equivalents of NaOAc (0.5 g, 6 mM) in 70° EtOH (12 ml). The soln was left at room temp. overnight. After concen, the aq. mixture was extracted with ET_2O. A white powder was obtained after crystallization from hexane–EtOAc, with 50% yield (0.2 g, 1 mM). Mp 116°; UV $\lambda_{max}^{H_2O}$ nm (log *ε*): 246 (2.73), 303 (4.06); ^1H NMR (CD_3OD): δ 0.75 (3H, *d*, *J* = 8 Hz, H-9), 0.96 (3H, *t*, *J* = 7 Hz, H-8), 1.3 (2H, *m*, H-7), 1.85 (H, *m*, H-6), 2.37 and 2.52 (3H, *s*, H-11); 35–65% (*Z*-*E*), 3.70 and 3.78 (H, *d*, *J*

= 3 Hz, H-5, 35–65%, *Z-E*); DCIMS (NH_3) 90 eV, *m/z* (rel. int.): 213 $[M+H]^+$ (100), 197 (12), 156 $[M+H-butyl]^+$ (7); (Found: C, 56.72; H, 7.55; N, 12.71. $C_{10}H_{16}N_2O_3$ requires: C, 56.6; H, 7.54; N, 13.21%.).

(5S, 6S)-3-[2-(*methyl-imino)ethyl*] 5-sec-*Butyl pyrrolidine*-2,4-*dione* (**16**). L-TA (0.4 g, 2 mM) was dissolved in MeOH (4 ml) with two equivalents of methylamine (0.35 ml of a 33% ethanolic soln). The mixture was refluxed for 2 hr and concd. Recrystallization from hexane–MeOAc (1:1) gave white crystals with 50% yield (0.2 g, 1 mM). Mp 116°; UV $\lambda_{max}^{H_2O}$ nm (log ε): 240 (4.0), 300 (4.27); ^{1}H NMR ($CDCl_3$): δ0.75 (3H, *d*, *J* = 7 Hz, H-9), 0.95 (3H, *t*, *J* = 7 Hz, H-8), 1.3 (2H, *m*, H-7), 1.93 (H, *m*, H-6), 2.54 and 2.56 (3H, *s*, H-11, 50–50%, *Z-E*), 3.02 and 3.08 (3H, *d*, H-13, 50–50%, *Z-E*), 3.70 (H, *m*, H-5); GCEIMS 70 eV, *m/z* (rel. int.): 210 $[M]^+$ (7), 154 $[M-56]$ (100); (Found: C, 62.12; H, 8.59; N, 12.45. $C_{11}H_{18}N_2O_2$ requires: C, 62.85; H, 8.57; N, 13.33%.).

(5S, 6S)-3-[2-(*Thiosemicarbazone-imino)ethyl*] 5-sec-*butylpyrrolidine*-2,4-*dione* (**17**). L-TA (0.2 g, 1 mM), was treated with one equivalent of thiosemicarbazide (0.1 g, 1 mM) in 70° EtOH (10 ml) and refluxed for 1 hr. Et_2O was added to the concd soln and the ppt. was recrystallized from 50° EtOH with a yield of 5% (0.14 g, 0.5 mM). mp 170°, lit. [31] 143–147°; ^{1}H NMR (CD_3OD): δ0.8 (3H, *t*, *J* = 7 Hz, H-8), 1.0 (3H, *d*, *J* = 7 Hz, H-9), 1.30 (2H, *m*, H-7), 1.85 (H, *m*, H-6), 2.5 (3H, *s*, H-11), 3.72 (H, *d*, *J* = 3 Hz, H-5), DCIMS (NH_3) 90 eV, *m/z* (rel. int.): 271 $[M+H]^+$ (100), 254 (8), 214 $[M+H-butyl]^+$ (14), 197 (40); (Found: C, 48.5; H, 6.71; N, 20.04; S, 11.68. $C_{11}H_{18}N_4O_2S$ requires: C, 48.88; H, 6.66; N, 20.74; S, 11.86%.).

Biological assays. Root growth inhibition. Sterilized rice seeds (variety Irat13 obtained from CIRAD-IRAT, Montpellier, France) were pregerminated in the dark, for 3 days at 28°. Ten germinations were selected at random and distributed in each Petri dish (150 mm diameter) containing 10 ml of the soln to be tested, buffered with 0.01 M MES, pH 6. An aliquot of 40 roots was measured with a ruler to calculate the initial mean root length. After 2 days at 28°, the root lengths were measured and the increase in length calculated. Each treatment (compound-dose) had three replicates (Petri dish) within each of three independent experiments. The dose-inhibition curve was estimated by linear regression with the following transformed variables: $Y = \text{arc}\sin(\sqrt{[I/100]})$ and $X = \log_{10}(M)$ where I was the growth inhibition percentage and M was the molar concentration of the compound [35]. ID_{80} and ID_{50} were estimated by interpolations of the linearised dose-inhibition curve.

Leaf toxicity and browning induction. All the analogues were tested on rice leaves of plants at the 5-leaf stage (25–30 days old) cultivated in a greenhouse (20° min). The varieties Irat 13 (TA highly reactive) and Maratelli (TA weakly reactive) [37] were used. The last developed leaves were kept flat and treated with 10 μl drops of the sodium salt of each analogue (pH 6, without buffer) at two concentrations, 10 mM and 3 mM. The whole plants were kept in a humid chamber for 24 h. In each experiment, TA was used as a control of the plant reaction since the browning capacity of the leaves varies with the season [37]. After 5–8 days, leaves were scored for their reaction according to the following scales. Browning: weak (+), intermediary (++), intense (+++). Necrosis: weak (small lesion: +), intense (large lesion: ++).

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REFERENCES

1. Kaczka, E., Gitterman, C., Dulanay, E., Smuth, M., Hendlin, D., Woodruff, H. and Folkers, K. (1964) *Biochem. Biophys. Res. Commun.* **14**, 54.
2. Gitterman, C., Dulaney, E., Kaczka, E., Campbell, G., Hendlin, D. and Woodruff, B. (1964) *Cancer Res.* **24**, 440.
3. Gitterman, C. (1964), *J. Med. Chem.* **8**, 483.
4. Miller, F., Rightsel, W., Sloan, B., Ehrlich, J., French, J., Bartz, Q. and Dixon, G. (1963) *Nature* **200**, 1338.
5. Cole, M. and Rolinson, G. (1972) *Appl. Microb.* **24**, 660.
6. Smith, E., Fredrickson, T. and Hadidian, Z. (1968) *Cancer Chemoth. Rep.* **52**, 579.
7. Giambrone, J., Davis, N. and Diener, V. (1978) *Poultry Sci.* **57**, 1554.
8. Meronuck, R., Steele, J., Mirocha, C. and Christensen, C. (1972) *Appl. Microb.* **23**, 613.
9. Mikami, Y., Nishijima, Y., Iimura, H., Suzuki, A. and Tamura, S. (1971) *Agric. Biol. Chem.* **35**, 611.
10. Iwasaki, S., Muro, H., Nozoe, S., Okuda, S. and Sato, Z. (1972) *Tetrahedron Letters* **1**, 13.
11. Umetsu, N., Kaji, J. and Tamari, K. (1972) *Agric. Biol. Chem.* **36**, 859.
12. Shigeura, H. and Gordon, C. (1963) *Biochemistry* **5**, 1132.
13. Umetsu, N., Muramatsu, T., Honda, H. and Tamari, K. (1974) *Agric. Biol. Chem.* **38**, 791.
14. Carrasco, L. and Vazquez, D. (1972) *J. Antibiot.* **25**, 732.
15. Muramatsu, T., Umetsu, N., Matsuda, K. and Tamari, K. (1974) *Agric. Biol. Chem.* **38**, 2049.
16. Carrasco, L. and Vazquez, D. (1973) *Biochem. Biophys. Acta* **319**, 209.
17. Barbacid, M. and Vazquez, D. (1974) *J. Mol. Biol.* **84**, 603.
18. Barbacid, M. and Vazquez, D. (1974) *Eur. J. Biochem.* **44**, 437.
19. Lacey, R. (1954) *J. Chem. Soc.* 850.
20. Harris, S., Fisher, L. and Folkers, K. (1965) *J. Med. Chem.* **8**, 478.
21. Yuki, H., Kaizu, Y. Yoshida, S., Higuchi, S., Honda, S. and Takiura, K. (1971) *Chem. Pharm. Bull.* **19**, 1664.
22. Yuki, H., Tsujimoto, T., Sawada, T., Takiura, K. and Yamaguchi, T. (1976) *Yakugaku Zasshi* **96**, 536.
23. Yamaguchi, T., Yosida, S., Higuchi, S., Sakamoto, Y., Saito, K. and Yuki, H. (1981) *Yakugaku Zasshi* **101**, 125.
24. Yuki, H., Kianaka, E., Yamao, A., Kariya, K. and Hashimoto, Y. (1971) *Gann* **63**, 199.
25. Hashimoto, Y., Ojhima, H. and Yuki, H. (1972) *Gann.* **63**, 79.
26. Arai, T. (1983) *FEBS Letters* **155**, 273.
27. Roach, M. C. and Luduena, R. F. (1985) *Biochem. Biophys. Res. Commun.* **129**, 200.
28. Bhat, S., Kohl, H., Ganguli, B. and Desouza, N. (1977) *Eur. J. Med. Chem.* **12**, 53.
29. Matsuo, K., Kitaguchi, I., Takata, Y. and Tanaka, K. (1980) *Chem. Pharm. Bull.* **28**, 2494.
30. Matsuo, K., Kimura, M., Kinuta, T., Takai, N. and Tanaka, K. (1984) *Chem. Pharm. Bull.* **32**, 4197.
31. Yuki, H., Tohira, Y., Aoki, B., Kano, T., Takama, S. and Yamazaki, T. (1967) *Chem. Pharm. Bull.* **13**, 1107.
32. Suzuki, S., Sano, F. and Yuki, H. (1967) *Chem. Pharm. Bull.* **15**, 1120.
33. Mikami, Y. and Nishijima, Y. (1972) *Nippon Nogei Kagaku Kaishi* **46**, 473.
34. Ibi, A., Taniguchi, E. and Maekawa, K. (1979) *Agric Biol. Chem.* **43**, 1641.
35. Lebrun, M., Duvert, P., Gaudemer, F., Gaudemer, A., Deballon, C. and Boucly, P. (1985) *J. Inorg. Biochem.* **24**, 167.
36. Stack, M., Mislivec, P., Roach, J. and Pohland, A. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 640.

37. Lebrun, M., Orcival, J. and Duchartre, C. (1984) *Rev. Cytol. Biol. Veget. Botanique* **7**, 249.
38. Suzuki (1965) in *Rice Blast Disease* (Ou, S. H. ed.) p. 277. Irri Press, Philippines.
39. Ou, S. (1985) in *Rice Diseases*, p. 174. Commonwealth Agricultural Bureaux Press, Kew, U.K.
40. Poncet, J., Jouin, P., Pantaloni, A., Castro, B., Nisato, D., Nicolas, L. and Gaudemer, A. (1987) *Helv. Chim. Acta* (in press).
41. Nolte, M., Steyn, P. and Wessels, P. (1980) *J. Chem. Soc., Perkin, I*, 1057.
42. Arpino, P. and Devant, G. (1979) *Analysis* **7**, 348.
43. Brenner Von, M. and Huber, W. (1953) *Helv. Chim. Acta* **34**, 1109.
44. Lacroix, E., Solomon, E., Mamalis, P. and Harrington, F. (1975) *Pest. Sci.* **6**, 491.
45. Schmidlin, T. and Tamm, C. (1980) *Helv. Chim. Acta* **63**, 121.
46. Jones, R. and Peterson, G. (1983) *Tetrahedron Letters* **24**, 4751.
47. Van der Bann, J., Barnick, J. and Bickelhaupt, F. (1978) *Tetrahedron* **34**, 223.
48. Mulholland, T., Foster, R. and Haydock, D. (1972) *J. Chem. Soc. Perkin I*, 2121.
49. Matsuo, K. and Tanaka, K. (1984) *Chem. Pharm. Bull.* **39**, 3724.