Antimutagenic unusual amino acids from plants[1]

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Summary. Five unusual amino acids were identified as antimutagens against spontaneous mutation of *Salmonella typhimurium* TA100: L-azetidine-2-carboxylic acid (**1**) from Liliaceae plants, α-(methylenecyclopropyl)glycine (**2**) from *Litchi chinensis* seeds, and 2-amino-4-methylhex-5-ynoic acid (**3**), hypoglycin A (**4**), and *(2S,4R)*-2-amino-4-hydroxyhept-6-ynoic acid (**5**) from *Euphoria longana* seeds. The absolute stereochemistry of **5** was determined by its chiral synthesis from L-allylglycine, proving that **5** is the C-4 epimer of the amino acid previously isolated from dried longan seeds.

Key words. Antimutagens; natural product; unusual amino acids; isolation and structural determination; Liliaceae; Sapindaceae; *Salmonella typhimurium* TA100.

Recently, environmental mutagens have become a serious problem because most of the potent mutagens identified with the aid of quick mutagenicity tests using microorganisms often proved to be synonymous with carcinogens. In general, mutation requires several steps after being triggered by mutagens; therefore, we can expect some modulating or antagonizing activities against mutagenesis at several of the different stages leading to fixation. The modulators which are effective in decreasing the mutant formation only at the fixation stages are called 'antimutagens'[3], as opposed to 'desmutagens', which prevent only the triggering of mutational events by chemical or physical elimination of the active mutagens.

Identification of antimutagens and other such modulators will provide useful tools for studying the mechanisms of both mutation and repair, and in some cases, it may give some insight into the prevention of carcinogenesis.

We have screened for naturally-occurring antimutagens employing the paper disk assay[4] with the strains *Salmonella typhimurium* TA100[5], *E. coli* WP2 and *B. subtilis* NIG1125 to identify several active extracts out of about 500 plant samples[4]. Based on these primary assay results, we have been performing isolation and characterization of the active principles in these plant extracts, and have isolated protoanemonin form *Ranunculus* and *Anemone* plants as the antimutagen against UV- and nitrosoguanidine-induced mutations of *E. coli* WP2[6].

In this paper, the isolation and characterization of five antimutagenic amino acids which were effective against spontaneous mutation of *S. typhimurium* TA100 will be discussed, including L-azetidine-2-carboxylic acid (**1**), α-(methylenecyclopropyl)-glycine (**2**), 2-amino-4-methylhex-5-ynoic acid (**3**), hypoglycin A (**4**), and *(2S,4R)*-2-amino-4-hydroxyhept-6-ynoic acid (**5**). Among these antimutagenic amino acids, **5** was proved to be the C-4 epimer of an amino acid present in dried longan seeds[7], by means of a 4-step chiral synthesis of both diastereomers.

The 80% aqueous methanol extract of aerial parts of lily-of-the-valley *(Convallaria majalis)* showed strong antimutagenic activities against spontaneous mutation of *S. typhimurium* TA100. The following isolation studies of the active factor was monitored by bioassay results: i.e., partitioning of the crude extracts with a chloroform-methanol-water system followed by treatment of the aqueous layer on the cation-exchange column of Diaion SK1B (H^+ form) and elution with H_2O, with 1 M and then with 3 M aqueous NH_4OH. The accumulated activity in the 1 M-NH_4OH eluate suggested that the active principle was an amino acid or peptide, or some analogue. Column chromatography of the 1 M-NH_4OH eluate on Sephadex G-10 (H_2O) followed by cation-exchange chromatography (MCI-Gel CK10H, 0.2 M-ammonium formate buffer, pH 3.15–3.55, linear gradient) and desalting yielded a pure active compound. Its physical data, especially ^{1}H-NMR (in D_2O) and mass spectrum, suggested that it was azetidine-2-carboxylic acid (**1**)[8,9], the most abundant and common unusual amino acid among Liliaceae plants[10]. In addition to *C. majalis*, we identified **1** in some other active plant extracts, such as *Ophiopogon japonicus, Liliope tawadae,* and *Polygonatum odoratum* var. *pluriflorum* (Liliaceae), and a mushroom *Clavaria miyabeana* (Clavariaceae)[11].

The extraction and isolation procedure for the active principle from fresh litchi (*Litchi chinensis,* Sapindaceae) seeds were similar to what was described above. The antimutagenic amino acid thus obtained was identified as α-(methylenecyclopropyl)glycine (**2**), which had previously been isolated from the same source[12]. The leaves and edible portion of litchi fruits were devoid of antimutagenic activity; this is in agreement with the report that no methylenecyclorpropylglycine could be detected in the leaves or fresh arils[12].

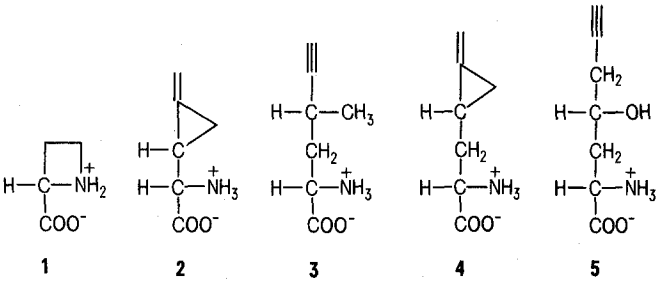
From longan (*Euphoria longana,* Sapindaceae) seed extract, we purified three antimutagenic amino acids: The most active and abundant component was 2-amino-4-methylhex-5-ynoic acid (**3**)[12,13] which had been previously isolated from dried longan seeds[7]. The second-most active component was shown to be a higher homologue of **2**; i.e. β-(methylenecyclopropyl)alanine, known as hypoglycin A[14,15], **4**. Hypoglycin A was first isolated from unripe ackee fruits (*Blighia sapida,* Sapindaceae) as a toxic constituent causing vomiting sickness and hypoglycemia in West Indies[14,16,17], and was subsequently isolated from *Billia hippocastanum* seeds (Sapindaceae)[18], but as yet had not been found in longan. The least active principle was a very minor component; only 4 mg was isolated from 247 g fresh seeds after a quick cation-exchange chromatography, $\Delta\varepsilon_{199}$+1.39 (H_2O). This component was very unstable under acidic or even neutral condi-

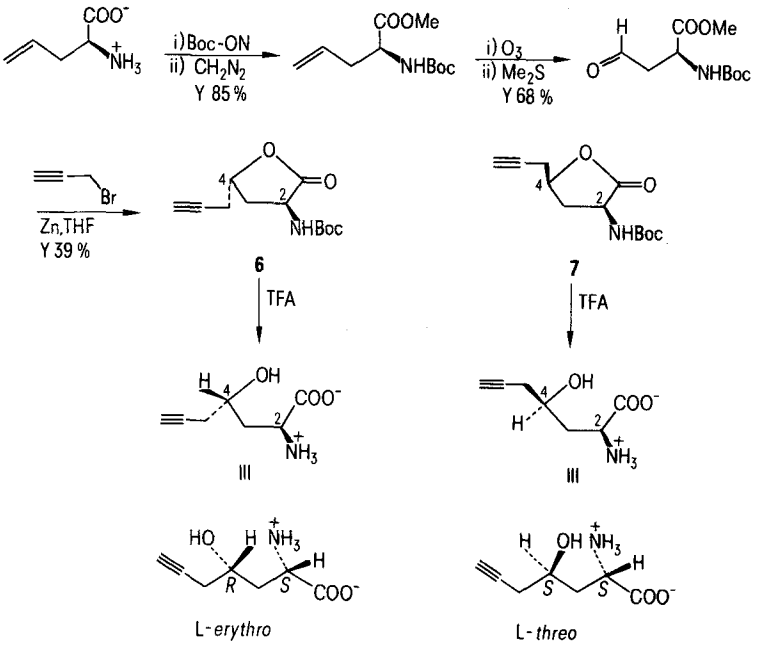
Antimutagenicity of amino acids 1–5 against spontaneous mutation of *Salmonella typhimurium* TA100[a]

Amino acids	Antimutagenic zone diameter[b] for 100 μg/disk	10 μg/disk
1	40 mm	0
2	38 mm	0
3	58 mm	34 mm
4	24 mm	0
5	< 10 mm	0

[a] Antimutagenic assays were performed using a paper disk method[4]: Paper disks (8 mm diameter) containing 100 μg and 10 μg of the compounds were placed on the minimal agar bed where the tester strain was applied. Incubation was performed at 37° for 2 days.

[b] Diameter of the zones where no mutant colonies were observed without inhibition of the background growth of the tester strain.





tions. ^{1}H-NMR studies of this compound revealed it to be 2-amino-4-hydroxyhept-5-ynoic acid, a structure which was already assigned to an amino acid isolated from dried longan seeds[7] with its stereochemistry unsolved.

In order to establish the structure including the absolute stereochemistry, we performed the synthesis of both diastereomers at the C-4 position starting from optically active L-allylglycine as follows (scheme). The protected diastereomeric lactones **6** and **7** were separated by reverse phase HPLC and their relative stereochemistries were determined by nuclear Overhauser effect (nOe) experiments; namely, a 2-H/4-H nOe was present in **6** but absent in **7**. Thus, the hydroxy amino acids derived from these two isomers after deprotection were L-*erythro*, $[\alpha]_D$+14.9° (c 0.8, H_2O), $\Delta\varepsilon_{197}$+0.91(H_2O), and L-*threo* isomers, $[\alpha]_D$ −22.6° (c 1.0, H_2O), $\Delta\varepsilon_{199}$+1.33 (H_2O), respectively.

Comparison of ^{1}H-NMR spectra (in D_2O, 360 MHz) of the antimutagenic amino acid with these synthetic isomers showed that it was the L-*erythro* isomer, i.e., *2S,4R*. It is noteworthy that the 2-amino-4-hydroxyhept-6-ynoic acid previously isolated from dried longan seeds[19], lit. $[\alpha]_D^{20}$ − 27° (c 2, H_2O)[7], $\Delta\varepsilon_{199}$+1.07, $\Delta\varepsilon_{195}$+1.41[13] had an identical ^{1}H-NMR spectrum with the L-*threo* isomer; i.e., *2S,4S*. The presence of both diastereomers in the crude amino acid preparations of both fresh and dried longan seeds were confirmed by an amino acid analyzer, the *erythro*/*threo* ratio being 1:4 and 1:2, respectively.

So far we have identified 5 unusual amino acids with antimutagenic activity against sponteneous mutation of *S. typhimurium* TA100, as shown in the table. Most of these amino acids can be considered as antimetabolites of amino acids found in proteins[20]; **1** for proline, and **2** through **5** for leucine, isoleucine, or valine. Perhaps these antimutagenic amino acids with unique structures have common modes of action, and differ from previously identified antimutagens which tend to be lipophilic and possess an active double bond; i.e., protoanemonin[6], cinnamaldehyde[21], coumarins[22], enmein, nodosin, oridonin[23]. In order to obtain some insights into their mechanism of action, co-application of the corresponding L-amino acid with each sample on paper disk assays was performed. However, no reduction of the antimutagenicity of these amino acids **1** through **5** was observed. Mechanistic studies of the antimutagenicity of these unusual amino acids including the antimetabolite problem are in progress.

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