2-AMINO-5-METHYL-5-HEXENOIC ACID, A METHIONINE ANALOG PRODUCED BY *STREPTOMYCES* SP. MF374-C4

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2-Amino-5-methyl-5-hexenoic acid (AMHA), a new methionine analog, was isolated from a fermentation broth of *Streptomyces* sp. MF374-C4 based on its reversal of the effect of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in a test system that determines the size of growth zones of revertants (His^+) of *Salmonella typhimurium* TA1535. AMHA also inhibited growth of the tester strain in a synthetic medium. These AMHA activities were abolished by methionine. The incidence of spontaneous streptomycin-resistant mutations of *Escherichia coli* K12 was not decreased by AMHA at concentrations where cell growth was partially inhibited. AMHA inhibited protein synthesis but not DNA or RNA synthesis in *S. typhimurium* TA1535 and *E. coli* K-12. The analog inhibited formation of methionyl-tRNA but not of valyl-tRNA in a cell-free system of *E. coli*, and supported ATP-PPi exchange in the cell-free system. At concentrations where it inhibited cell growth, AMHA decreased the number of foci, induced by ROUS sarcoma virus, on cultured sheets of chick-embryo fibroblasts. The effects of AMHA on focus formation and on the cell growth were overcome by methionine.

Since most carcinogens are mutagens and *vice versa* we have been seeking anti-carcinogens among fermentation products of streptomyces by determining their possible antimutagenic activity in a bacterial mutation system, by determining the effects of test samples on either the size of growth zones or the number of colonies of revertants of *Salmonella typhimurium* TA1535 induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). A fermentation product of *Streptomyces* sp. MF374-C4 was isolated based on its reversal of the action of MNNG in this test and its structure was determined to be L-2-amino-5-methyl-5-hexenoic acid (AMHA), a methionine analog.

Analysis of its biological and biochemical activities indicated that AMHA primarily inhibited protein synthesis, competing with methionine and resulting in inhibition of cell growth. Its apparent antimutagenic effect could be a reflection of partial inhibition of cell growth.

Materials and Methods

Test for Possible Antimutagenic Substances

Agar plates for quantitative testing of mutagens were prepared essentially as described by AMES *et al.*[1] with *S. typhimurium* TA1535 as the tester strain. N-Methyl or -ethyl-N′-nitro-N-nitrosoguanidine (MNNG or ENNG), chosen as standard mutagens throughout these experiments, were dissolved in methanol at desired concentrations and 50 μl portions of the solution were added to paper discs (8 mm in diameter), which were then dried at room temperature. These were placed on agar plates containing 10 ml of a bottom agar layer of 1.5% agar in VOGEL-BONNER medium E supplemented with 2% glucose,

520 μg/liter of biotin, and 810 μg/liter of histidine; and 2.5 ml of a top agar layer containing 7×10^7 cells suspended in a 0.6% agar solution in water. After incubation at 37°C for 48 hours, growth zones of revertants (His^+) were measured (Fig. 1). Antimutagenic substances were estimated by their activity in decreasing the growth zones of revertants induced by MNNG (or ENNG). To accomplish this, paper discs were first soaked in solutions to be tested and dried at room temperature before receiving MNNG (or ENNG). The bactericidal activity of a test compound could be distinguished by a sterilized zone on the light lawn of the background. ENNG was kindly supplied by Prof. TAIJIRO MATSUSHIMA, Institute of Medical Science, University of Tokyo, and MNNG and *S. typhimurium* TA1535 by Dr. TAKASHI SUGIMURA, National Cancer Center Research Institute.

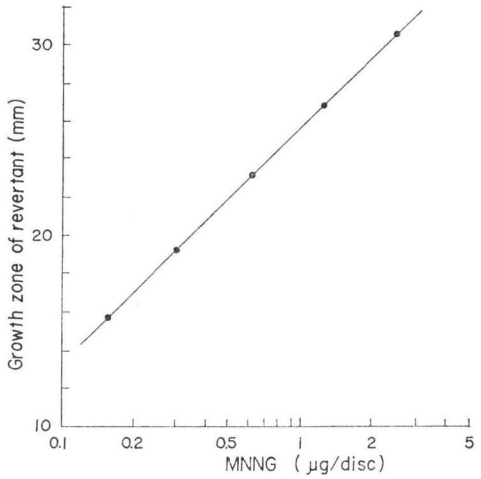
Results and Discussion

Fermentation, Isolation and Structural Studies

AMHA was produced by rotary-shaking a culture of *Streptomyces* sp. MF374-C4 at 27°C for 4 days in medium containing 1.5% glycerol, 1.5% cotton seed meal, 0.2% L-asparagine, and 0.2% NaCl. The initial pH of the medium had been adjusted to 7.4 with 1 N NaOH. The fermentation broth was brought to pH 2, centrifuged, and the supernatant fluid (4.5 liters) was applied to a column of Dowex 50W-X4 (a mixture of 120 ml of H^+ form and 280 ml of NH_4^+ form, 50~100 mesh). The column was washed with water and eluted with 0.5 N NH_4OH. The eluted fractions were applied to paper discs, which were dried free of NH_4OH and examined for reversal of the action of either MNNG or ENNG as described above. Active fractions were combined and dried *in vacuo*. The dried material was dissolved in 100 ml of a buffer (0.1 M pyridine-formic acid, pH 3.1) and applied to a 350 ml column of Dowex 50W-X8 (100~200 mesh) equilibrated with the same buffer. The column was washed with the same buffer

Fig. 1. The relationship between the amount of MNNG and the growth zone of revertants of *S. typhimurium* TA1535.

The numbers on the paper discs show the amount of MNNG (μg/disc). The diameter of the growth zones of revertants was a linear function of log concentrations of MNNG. For details, see Methods.



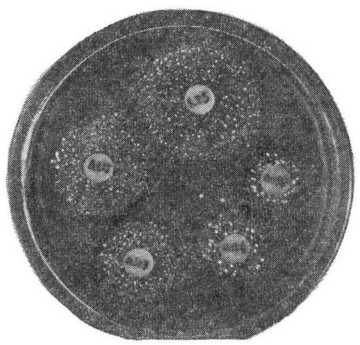
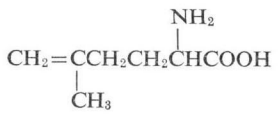
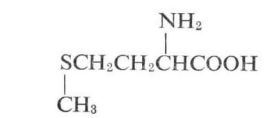


Fig. 2. Structures of L-2-amino-5-methyl-5-hexenoic acid and methionine.



L-2-Amino-5-methyl-5-hexenoic acid



Methionine

and eluted with 0.2 M pyridine-formic acid (pH 3.1). Active fractions were combined (750 ml) and applied to a 80-ml column of Dowex 50W-X4 (H^+). The column was washed with water and eluted with 0.5 N NH_4OH. Active fractions were combined, concentrated *in vacuo*, and left standing in a refrigerator. Colorless plates of AMHA were collected by filtration and dried *in vacuo*, yielding 346 mg in one experiment.

Physical and chemical properties of AMHA are as follows: melting range, 200~249°C (decomp.); $[\alpha]_D^{22}$ +34° (*c* 1.0, 0.5 N HCl); positive COTTON effect at 225 nm in 0.5 N HCl; pKa′ at 2.3 and 9.7 (titration equivalent, 147); positive color reactions with $KMnO_4$ and ninhydrin; ultraviolet absorption towards the end of short wave lengths; IR ν_{max} cm^{-1} (KBr) at 3450, 3100, 2950, 2600, 2100, 1585 (broad), 1515, 1450, 1410, 1355, 1325, 1200, 1150, 1080, 925, 885 (>C=CH_2), 850, 800 and 755. The formula $C_7H_{13}NO_2$ (M.W., 143.18) was shown by elementary analysis (Calcd: C 58.72, H 9.15, N 9.78, O 22.35, Found: C 58.60, H 9.12, N 10.00, O 22.38) and by the titration equivalent. The results of ^{1}H- and ^{13}C-NMR analysis are shown in Fig. 2 and Table 1. Catalytic hydrogenation of AMHA afforded colorless plates melting at 238~245°C (decomp.), identified as L-2-amino-5-methylhexanoic acid[2] by IR spectroscopy. Treatment of AMHA with acetic anhydride in methanol at room temperature for 20 hours gave 2-acetamide-5-methyl-5-hexenoic acid (m.p. 117°C) and its methyl ester (m.p. 83°C). Mass spectroscopy of the latter compound showed *m/e* 200 ($M^+ + 1$) and *m/e* 168 ($M^+ - 31$). ^{1}H-NMR analysis (dioxane-d_8, TMS) of the compound showed CH and NH at δ 4.45 and 6.95 ppm ($J_{CH,NH}$ = 8.5 Hz), respectively and OCH_3 and CH_3CO at δ 1.89 and δ 1.72 ppm. The positive COTTON effect at 225 nm indicates that C-2 is in L-configuration[3]. The disappearance of the 885 cm^{-1} band of IR

Table 1. The chemical shifts in ^{1}H- and ^{13}C-NMR of AMHA.

Proton*	Chemical shifts (δ)	Carbon**	Chemical shifts (δ)
		1	172.9*s*
2-H	4.42 *t*	2	53.4*d*
3-H_2	2.45~2.96*m*	3	28.74*t****
4-H_2		4	33.03*t****
		5	145.5 *s*
6-H_2	5.51 *m*	6	111.9 *t*
7-H_3	2.45 *d*	7	22.2 *q*

* Chemical shifts were measured in D_2O at 50°C using TMS as the external reference (100 MHz). Abbreviations of *d*, *t* and *m* indicate multiplicity of doublet type, triplet type and multiplet type, respectively. Complicated long range spin-spin coupling was observed in all signals.

** δ stands for ppm from TMS using dioxane (δ=67.4 ppm) as the internal reference and the chemical shift was determined in D_2O at pH 1.0. At pH 5.7***, C-3 and C-4 shifted +0.8 ppm and +0.3 ppm, respectively. Abbreviations of *s*, *d*, *t* and *q* indicate multiplicity of singlet, doublet, triplet and quartet, respectively, in an experiment of off-resonance.

Table 2. The number of colonies of revertants[a] or of the original strain[b] found on agar-plates containing various concentrations of AMHA, with[a] or without[b] ENNG.

AMHA (μmole/plate)	(a) Mutant (His^+)	(b) The tester strain (His^-)
0	1164/plate	599/plate
0.019	1036	610
0.038	1029	599
0.075	800	479
0.15	390	27
0.3	0	12

0.06 μmole ENNG/plate

The experiment was conducted by the method of AMES *et al.*[1] with the following modification. AMHA at various concentrations and ENNG at 0.06 μmole/plate were dissolved in the top agar (a). ENNG was absent from the top agar while the amount of histidine was increased to 3.9 mM (b). The tester strain (*S. typhimurium* TA1535) was precultured in nutrient broth until 3×10^8 cells/ml was reached. The cell suspension was diluted with the top agar to 3×10^7 cells/plate (a) or 6×10^2 cells/plate (b).

upon reduction and the ^{13}C-chemical shifts of C-5 and C-6 indicate that C-5 and C-6 make a vinyl group. All these data support the structure of L-2-amino-5-methyl-5-hexenoic acid.

Is AMHA truly Counteracting Mutagenesis in *S. typhimurium* TA1535?

AMHA was isolated from fermentation broths based on its activity in decreasing the size of growth zones of MNNG-induced revertants (His^+). When AMHA was applied to paper discs at increasing concentrations in addition to a fixed concentration of MNNG, the size of growth zones became smaller; 1.0 μg/ml of MNNG alone (23.0 mm), plus AMHA at 0.78 μg/ml (22.5 mm), 1.56 μg/ml (17.0 mm), and 3.13 μg/ml (12 mm). Since the antibiotic was not effective if sufficient methionine were present in the test system, the problem remained as to whether AMHA was truly antimutagenic against alkylating agents or simply inhibited cell growth by competing with methionine. As Table 2 shows, AMHA lowered the number of mutants as well as that of the original cells at similar concentration ranges. Both effects were abolished by the addition of methionine but not of other amino acids (data not shown). These results led us to suspect that AMHA simply inhibited cell growth as an antimetabolite of methionine.

Fig. 3. Effect of AMHA on the synthesis of macromolecules in *S. typhimurium* TA1535.

S. typhimurium TA1535 was grown in VOGEL-BONNER'S minimal medium E supplemented with 2% glucose, 520 μg/liter of biotin, and 810 μg/liter of histidine, at 37°C with shaking until an early exponential growth stage ($A_{600}=0.036$) at which time deoxyadenosine was added to a final conc. of 2×10^{-4} M. For estimation of the synthesis of cellular macromolecules, an assay mixture consisted of 50 μl of the cultured cell suspension, 10 μl of a water solution of a radioactive precursor (see below), a water solution of AMHA at the desired concentration, and water to 100 μl final total volume. For determination of DNA, RNA and protein syntheses, 33 mμCi of ^3H-thymidine (1.5 Ci/m mole), 33 mμCi of ^3H-uridine (17.7 Ci/m mole), and 200 mμCi of ^{14}C-valine (239 mCi/m mole) were added to assay mixtures, respectively. The mixtures were incubated at 37°C for 20 minutes and 90 μl portions were transferred to paper discs with which cold (for DNA and RNA) or hot (for protein) trichloroacetic acid-insoluble radioactivity was determined.

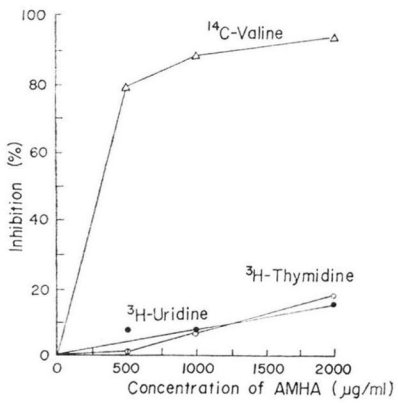


Table 3. ATP-PPi exchange dependent on AMHA.

Conditions		ATP formed (dpm)
AMHA	10^{-4} M	1,579
	10^{-3} M	3,236
	10^{-2} M	10,554
Methionine	10^{-6} M	1,384
	10^{-5} M	6,994
None		0 (2,485*)

ATP-PPi exchange dependent on AMHA. A reaction mixture contained in 250 μl, 0.1 M Tris·HCl, pH 7.5, 2 mM ATP, 2 mM $Na_4{}^{32}P_2O_7$ (0.05 μCi), 10 mM $Mg(OAc)_2$, 10 mM KCl, 25 mM KF, 0.08 mg of amino acid-RNA ligase[4], and the desired amount of an amino acid or of AMHA. After incubation at 37°C for 10 minutes, the reaction was terminated by mixing with 250 μl of cold 10% trichloroacetic acid. To the mixture, 0.1 ml of 7.5% (w/v) suspension of Norit A was added and, after standing at room temperature for 45 minutes, the Norit was filtered on a Whatman GF/C disc (2.5 cm diameter), washed with 5 ml water 5 times, dried and assayed for radioactivity with scintillation solution (0.4% Omnifluor in toluene) in a liquid scintillation counter.

When no amino acid was added to the reaction mixture a value 2,485 dpm was obtained as a blank. Estimates of ATP formed was obtained by subtracting this value from that of each sample.

Inhibition of Protein Synthesis by AMHA in Cells of *S. typhimurium* TA1535

Because of the structural resemblance between AMHA and methionine, the antibiotic was tested as an antimetabolite of methionine in protein synthesis. As Fig. 3 shows, inhibition of protein synthesis but not of DNA or RNA synthesis occurred in cells of *S. typhimurium* TA1535 incubated with increasing concentrations of AMHA.

Inhibition of Synthesis of Methionyl-tRNA by AMHA in a Cell-free System of *E. coli*

A similar selective inhibition of protein synthesis was obtained with *E. coli* (data not shown). Since AMHA was effective only when methionine was under limited supply, the interaction between them should be present in some step of protein synthesis. Although AMHA supported the ATP-PPi exchange reaction as do natural amino acids (Table 3), it inhibited synthesis of methionyl-tRNA but not of valyl-tRNA (Fig. 5). However, AMHA-$tRNA^{met}$, if formed, may not substitute for met-$tRNA^{met}$ in peptide bond formation considering the counteraction between AMHA and methionine in respect to cell growth. It may also be possible that proteins containing AMHA residues in the place of methionine residues, if formed, are not functioning. AMHA did not support the growth of *E. coli* P4X (Met B^-) on minimal agar but rather inhibited it in competition with methionine (data not shown).

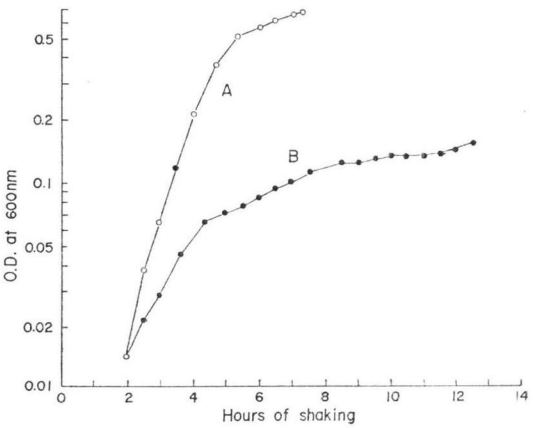
Effect of AMHA on Spontaneous Mutation in *E. coli*

During normal growth of *E. coli*, spontaneous streptomycin-resistant mutants appear at a low frequency. We wondered if AMHA could lower the frequency, in other words, the ratio of mutation to cell division. Although it partially inhibited cell growth, AMHA did not decrease but rather increased the frequency of spontaneous mutations (Fig. 4). If the partial inhibition of cell growth by AMHA is reflected by the balance of growth and possible death of cells, the actual number of cell divisions in the presence of AMHA should be larger than that which is calculated from the numbers of cells before and after the incubation. The low stimulation of spontaneous mutations by AMHA may be explained by this possibility.

Fig. 4. Effect of AMHA on the frequency of spontaneous mutations in *E. coli*.

E. coli K12 were harvested at an early exponential growth stage (A_{600}=0.13, in nutrient broth). The test culture contained 6.65 ml of a suspension of 4×10^6 cells in nutrient broth and 0.35 ml of AMHA solution (2 mg/ml in water). The control culture contained water instead of the AMHA solution. After incubation at 37°C under shaking for the indicated time (7.25 and 12.5 hours for the control run and the test run, respectively), appropriate portions of each culture were submitted to colony counting on nutrient-agar plates (for total viable cells) and on plates of nutrient-agar containing streptomycin at 2.5 μg/ml (for streptomycin-resistant cells).

A: without AMHA. B: with AMHA.



	At the growth stages of	
	A	B
Number of cells/culture		
Total	2.1×10^{10}	2.4×10^{9}
Str^r	14630	2270
Ratio of Str^r/Total	7×10^{-7}	9×10^{-7}

Fig. 5. Effect of AMHA on the synthesis of amino-acyl-tRNA *in vitro*.

A reaction mixture contained in 100 μl, 100 mM Tris·HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 10 mM KCl, 2 mM ATP, 0.2 mg of *E. coli* tRNA, either 0.005 μCi of ^{14}C-methionine (49 mCi/m mole) or 0.02 μCi of ^{14}C-valine (239 mCi/m mole) 0.032 mg of amino acid-RNA ligase[4], and the desired amount of AMHA. The reaction mixture without label (90 μl), was equilibrated at 37°C for 4 minutes then mixed with ^{14}C-amino acid solution (10 μl) and incubated for 6 minutes. A 90 μl portion of the mixture was transferred to a paper disc and its cold trichloroacetic acid-insoluble radioactivity was determined. Control runs to which no AMHA was added gave 4,580 dpm and 5,980 dpm with ^{14}C-methionine and ^{14}C-valine, respectively.

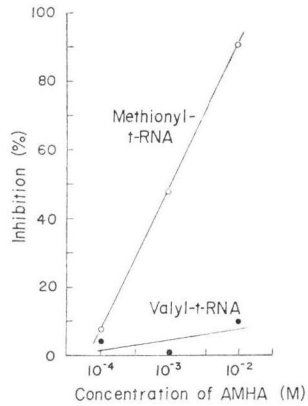


Table 4. Effect of AMHA on focus formation induced by the SCHMIDT-RUPPIN ROUS sarcoma virus in cultures of chick embryo fibroblasts.

AMHA (μg/ml)	No. of focus/plate	% Inhibition
0	204	—
125	176	14
250	170	16
500	120	41

Chick embryo fibroblasts (CEF) were cultured as described by RUBIN[5] in medium containing of 84.5% (v/v) EAGLE's MEM, 5% calf serum, 10% tryptose phosphate broth (Difco) and 0.5% chick serum[5]. CEF collected from the primary culture were infected with the SCHMIDT-RUPPIN strain of ROUS sarcoma virus (SR-RSV) by the method of SIMINOFF and REED[6] with minor modifications as follows: In each Petri dish (60 mm diameter), a 4-ml suspension of 1×10^6 CEF with 200 focus forming units of SR-RSV was placed and incubated at 37°C for 6 hours in a humidified atmosphere of 5% CO_2. The culture fluid was replaced by 7 ml of the medium described above prepared by dissolving 0.85% (w/v) Bacto agar (Difco) and a test sample at the desired concentration. Incubation was resumed and continued until day 8 when the cell sheet with agar-overlayer was fixed, the agar-overlayer was removed, and cells were stained for counting foci.

Effect of AMHA on Focus Formation of Chick Embryonic Cells Induced by ROUS Sarcoma Virus

The counteraction displayed by AMHA and MNNG (or ENNG) in the bacterial mutation experiment prompted us to test the possible anticarcinogenic activity of AMHA. As a test system, the transformation of chick embryonic fibroblasts induced by ROUS sarcoma virus was chosen. It is only hypothetical, however, that neoplastic processes induced by chemical carcinogens and by tumor viruses have a common molecular mechanism. As Table 4 shows, AMHA at a concentration as high as 500 μg/ml appeared to partially inhibit transformation as reflected by a decrease in the number of foci formed. However, here again, it was suspected that AMHA really did not inhibit the transformation process. A separate experiment indicated that AMHA at the same concentration inhibited growth of chick embryonic fibroblasts almost completely, leaving their viability unaffected (data not shown). The effect on focus formation as well as on cell growth was abolished by raising the concentration of methionine in the medium (data not shown). Therefore, it is possible that AMHA simply inhibited growth of the cells either before or after the transformation or both. Additional experiments testing the effect of AMHA on *in vivo* carcinogenic processes will answer if AMHA acts as a true anti-carcinogen.

Acknowledgement

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