Brief Articles

N-Terminal Dipeptides of D(−)-Penicillamine as Sequestration Agents for Acetaldehyde

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Since acetaldehyde (AcH), a toxic oxidation product of ethanol, may play an etiologic role in the initiation of alcoholic liver disease, we had earlier pioneered the development of *β*,*β*-disubstituted-*β*-mercapto-α-amino acids as AcH-sequestering agents. We now report the synthesis of a series of N-terminal dipeptides of D(−)-penicillamine, prepared from the synthon 3-formyl-2,2,5,5-tetramethylthiazolidine-4*S*-carboxylic acid (**3**), a cyclized N-protected derivative of D(−)-penicillamine. These dipeptides were equally or more effective than penicillamine in trapping AcH in a cell-free system. In experiments using a hepatocyte culture system, two of the dipeptides, D-penicillamylglycine (**6a**) and D-penicillamyl-*β*-alanine (**6d**), at 1/20 the molar concentration of ethanol, lowered the concentration of ethanol-derived AcH by 79% and 84%, respectively, at 2 h. The presence of cyanamide (an inhibitor of aldehyde dehydrogenase) in the incubation medium resulted in a 45-fold increase in ethanol-derived AcH; nevertheless, dipeptides **6a** and **6c** (D-penicillamyl-α-aminoisobutyric acid) were able to reduce this AcH level by approximately one-third.

Introduction

Ethanol metabolism produces acetaldehyde (AcH), a toxic oxidation product that may play an etiologic role in the initiation of alcoholic liver disease (ALD). Of pertinence in this regard, chronic alcoholics have higher blood AcH levels compared to nonalcoholics after consuming alcoholic beverages[1] and are, therefore, more susceptible to its toxic effects. It is now well-established that this ethanol-derived AcH binds covalently to cellular proteins, and such AcH-modified proteins can elicit an immune response manifested by antibody formation to these AcH-bound epitopes.[2] In rodents treated with ethanol, AcH binds to hemoglobin[3] and other plasma proteins,[4] to tubulin,[5] and to a specific cystolic liver protein,[6] as well as to liver collagen.[7] AcH−protein conjugates of hemoglobin[8,9] have also been detected in humans after consumption of alcohol. The immunological consequences of AcH−protein binding[10] and its possible etiology in ALD[11] have been discussed.

L-Cysteine as well as L-cysteinylglycine have been reported to protect against AcH toxicity in vivo.[12] However, we found that while L-cysteine, which readily condenses with AcH to a cyclic thiazolidine-4-carboxylic acid at physiological pH, was a good AcH-trapping agent in vitro, it was totally ineffective in vivo due to its rapid catabolism.[13]

On the basis of the premise that AcH may play an etiologic role in alcohol-related diseases, we pioneered the development of AcH sequestration agents.[14] Our structure−activity studies suggested that the ideal AcH-sequestering agent should have (a) an acidic functional group to impart H_2O solubility and (b) 1,2- or 1,3-disubstitution with functional groups that can be sulfhydryl, amino, or hydroxyl. Of the trifunctional compounds tested that met these requirements, only *β*-mercapto-α-amino acids with one or two bulky substituents at the *β*-position were effective in sequestering AcH in vivo. Thus, D(−)-penicillamine (**1**) and *β*,*β*-tetramethylene-DL-cysteine (**2**) (Chart 1) were the best *β*-mercapto-α-amino acid sequestration agents for AcH in vivo.[13]

Although **1** is used clinically to treat Wilson's disease and hereditary cystinuria and to treat rheumatoid arthritis refractory to conventional therapy, its potential for renal and hematological toxicity[15] limits its usefulness as a sequestration agent for AcH. Accordingly, N-terminal dipeptides of **1** with glycine, *β*-alanine, L-valine, and α-aminoisobutyric acid (AIB) were projected for synthesis as potential AcH-sequestering agents with possibly attenuated toxicity. These dipeptides were designed such that one of the C-terminal amino acids (*β*-alanine) was of nonprotein origin, while another (AIB) was a sterically hindered, synthetic α-amino acid. Since the chiral center of **1** is opposite to that of the naturally occurring L-cysteine, these dipeptides would not be expected to be cleaved by endogenous peptidases, as is the case for L-cysteinylglycine or L-cysteinylvaline.[13]

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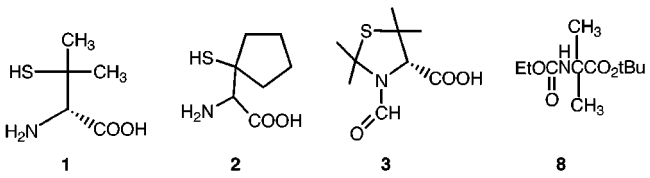
Chart 1



1 2 3 8

Results

The synthesis of N-terminal D(−)-penicillamine dipeptides required the synthon 3-formyl-2,2,5,5-tetramethylthiazolidine-4*S*-carboxylic acid (**3**; Chart 1), a cyclized N-protected derivative of **1** patterned after the corresponding L-cysteine derivative,[16] for peptide coupling reactions. The syntheses of D-penicillamylglycine (**6a)**, D-penicillamyl-L-valine (**6b)**, and D-penicillamyl-AIB (**6c**) are outlined in Scheme 1. Thus, the *tert*-butyl esters of glycine (**4a)**, L-valine (**4b**), and α-aminoisobutyric acid (**4c**) were coupled with the mixed anhydride prepared from **3** and isobutyl chloroformate in the presence of Et_3N to give the protected dipeptide esters **5**. Simultaneous deformylation, thiazolidine ring opening, and deesterification at the carboxy terminus were readily accomplished by heating **5** in 1 N HCl to give **6a, 6b**, and **6c** as their respective hydrochloride salts. For D-penicillamyl-*β*-alanine (**6d)**, the coupling procedure was identical to that described in Scheme 1, except that the commercially available ethyl *β*-alaninate (**4d**) was used to prepare the N-protected thiazolidine dipeptide ethyl ester **7**. Deblocking as above gave **6d.**

It is noteworthy that in the coupling of the *tert*-butyl ester of the sterically hindered AIB with the mixed anhydride of the equally sterically hindered **3,** the major product isolated was the N-carbethoxylated amino ester **8** (Chart 1), where the amino group of the amino ester had attacked the sterically less-hindered carbonyl group of the mixed anhydride. Thus, the yield of the desired product **5c** was commensurately low for this reaction.

The sequestration of AcH by the four N-terminal dipeptides of **1** in a cell-free system is shown in Figure 1. It can be seen that these dipeptides were equally or more effective than **1** itself (positive control) in trapping AcH. As predicted, methionine (Met), threonine (Thr), and serine (Ser) were totally without AcH-sequestering activity. In agreement with our previous results, L-homocysteine (Hcy) was comparable to **1** in sequestering AcH in vitro; however, Hcy was shown *not* to be an effective AcH-trapping agent in vivo.[13]

In experiments using a hepatocyte culture system,[17] D-penicillamylglycine (**6a)** and D-penicillamyl-*β*-alanine (**6d**), at 1/20 the molar concentration of ethanol (20 mM), lowered the concentration of ethanol-derived AcH by 79% and 84%, respectively, at 2 h (Figure 2A). We are unable to explain the anomalous result with compound **6c**. The presence of cyanamide (an inhibitor of AlDH[18]) in the incubation medium resulted in a 45-fold increase in ethanol-derived AcH (Figure 2B, control without dipeptide), and while **6a** and **6c** were able to reduce this concentration by 33% and 37%, repectively, it is clear that considerably higher dipeptide concentrations would be required under the latter conditions for effective AcH sequestration. Additional in vivo work in rodents is necessary to assess the degrees of efficacy, or toxicity, if any, of these compounds. Nevertheless, the present results suggest that selected N-terminal D-penicillamyl dipeptides can sequester ethanol-derived AcH in a manner comparable to that of D-penicillamine.

Discussion

Approximately 30−50% of Orientals lack a functional low K_m hepatic mitochondrial aldehyde dehydrogenase (AlDH2).[19] This enzyme plays a major role in the metabolic disposition of ethanol-derived AcH to acetate, this conversion serving as a detoxication mechanism for AcH. Those individuals lacking AlDH2 drink alcohol sparingly or not at all because of the consequence of experiencing a flushing syndrome, manifested by elevated blood AcH, facial flushing, tachycardia, and a general feeling of malaise reminiscent of the disulfiram−ethanol reaction,[20] on even moderate consumption of alcoholic beverages.[21]

By genotyping individuals for AlDH2, an indication of AcH exposure after ethanol consumption can be deduced.[22] The observation that a higher risk factor for esophogeal cancers exists among heavy drinkers with a mutant AlDH2 allele underscores the importance of AcH as a possible etiological factor in this disease.[23] Individuals with this heritable, mutant AlDH2 allele who refrain from drinking are generally protected from alcoholic liver disease as well as alcohol-induced cancers, whereas excessive drinking by subjects with this mutant allele places them at risk of developing not only esophogeal cancer but also cirrhosis of the liver. If total abstinence from alcohol cannot be voluntarily sustained, we envision the potential use of AcH-sequestering agents to protect chronic alcoholics, as well as those individuals with a nonfunctional AlDH2, from acetaldehydemia and its adverse pathologic consequences.

Other uses of these aldehyde-sequestering agents can also be envisioned. For example, methanol is metabolized in humans to formaldehyde and then to formic acid, and while metabolic acidosis elicited by the latter is a major problem, blindness induced by methanol is believed to be due to retinal destruction by formaldehyde.[24] Sequestration of methanol-derived formaldehyde[25] should, therefore, provide protection against methanol-induced blindness. Aldehyde sequestration agents may also be applicable (a) for the treatment of accidental overexposure in the workplace to toxic aldehydes, such as formaldehyde and glutaraldehyde, or (b) to counteract the toxic effects of AcH when subjects being treated for alcoholism with disulfiram, an inhibitor of AlDH, accidentally ingest alcohol.

Experimental Section

^{1}H NMR spectra were recorded at ambient temperature on either a GE-300 or a Bruker AC-200 NMR spectrometer. Chemical shifts are reported as *δ* values (ppm). Mass spectra (EI or FAB) were obtained on a Kratos MS 25 or Finnegan LCQ mass spectrometer. For TLC analyses, Analtech silica gel GF plates were used. The solvent systems used for TLC were as indicated. The plates were visualized by spraying with ninhydrin or ceric sulfate solution and heating. Column chromatography was carried out using columns packed with Kieselgel 60 (230−400 mesh) silica gel (EM Science). A rotating evaporator was used to remove solvents in vacuo.

3-Formyl-2,2,5,5-tetramethylthiazolidine-4*S*-carboxylic Acid (3). A suspension of 59.7 g (400 mmol) of D(−)-penicillamine in 1200 mL of acetone was heated under reflux with mechanical stirring for 2 h. The solids were allowed to

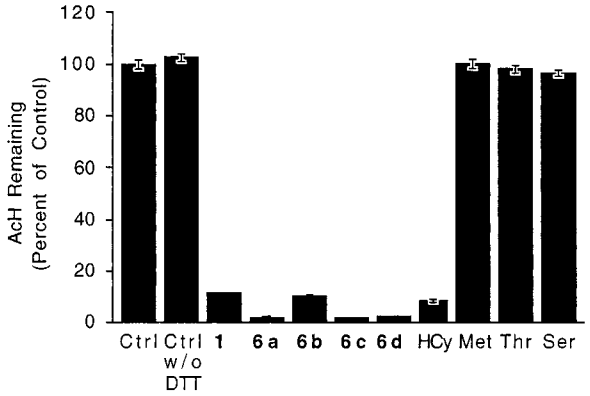


Figure 1. Sequestration of AcH by N-terminal dipeptides of D(−)-penicillamine and selected amino acids in a cell-free in vitro system. See Experimental Section for details.

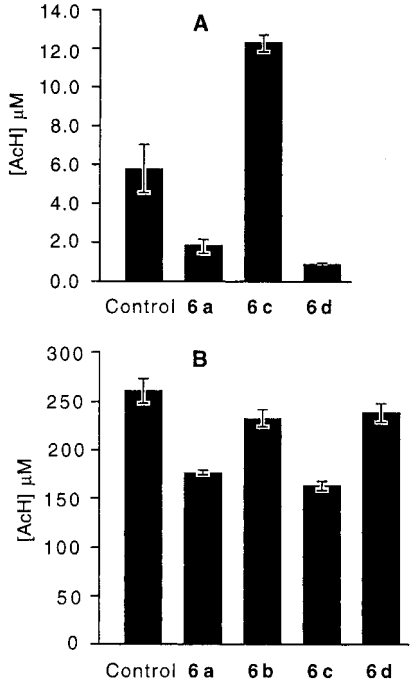


Figure 2. A. Effect of N-terminal D(−)-penicillamine dipeptides on AcH levels at 2 h in rat hepatocytes incubated with 20 mM ethanol and 1 mM dipeptide; $N = 4$. AcH was undetectable in controls without ethanol (not shown). B. Same as in A, except that cyanamide (50 μM), an inhibitor of AlDH, was added to the system.

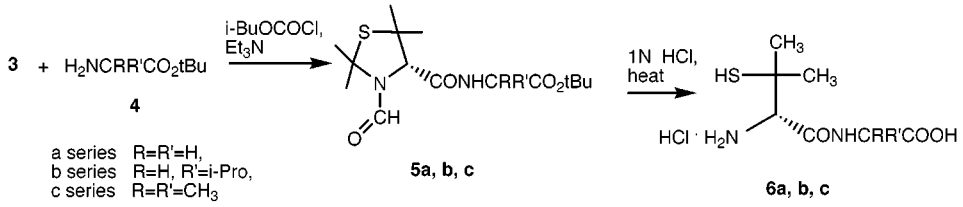
settle, and the supernatant solution was decanted through a filter. The residual solids were heated again in 400 mL of fresh acetone until complete solution resulted (5-10 min). The hot solution was poured through the same filter. This process dissolved most of the solids previously held on the filter. Cooling the combined filtrates resulted in the precipitation of a mass of solids which were collected. Concentration (by distillation of the acetone) of the mother liquor followed by cooling gave two additional crops of product giving a total of 68.4 g (90% yield) of 2,2,5,5-tetramethylthiazolidine-4*S*-carboxylic acid. This was used directly for formylation as described for the racemic compound.[26] Compound **3**, obtained in 69% yield, had a mp of 180−181 °C (lit.[26] mp 179−180 °C).

***N*-(3-Formyl-2,2,5,5-tetramethylthiazolidinyl-4*S*-carbonyl)-*β*-alanine Ethyl Ester (7).** To a solution of **3** (2.17 g, 10.0 mmol) in 50 mL of dry CH_2Cl_2 was added dry Et_3N (2.80 mL, 2.03 g, 20.1 mmol), and the solution was stirred under N_2 with ice bath cooling. To this solution were added isobutyl chloroformate (1.30 mL, 1.37 g, 10.0 mmol) and an additional 25 mL of CH_2Cl_2. The clear solution was stirred ca. 1 h, and *β*-alanine ethyl ester hydrochloride (1.56 g, 10.1 mmol) was added. The reaction mixture was allowed to warm to room temperature overnight and was then washed successively with 1 N HCl, 5% $NaHCO_3$, and water (50 mL of each). The solution was dried and the solvent evaporated to dryness in vacuo to give 2.89 g of viscous, colorless oil. This was purified by flash chromatography using EtOAc:hexane (1:4, 2:3, and 3:2) as eluent to give 2.12 g (67% yield) of **7** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.28 (t, $J = 7.1$ Hz, 3H, CH_3CH_2), 1.42, 1.44 (s, 1:6, 3H, CH_3CCH), 1.64, 1.69 (s, 7:1, 3H, CH_3CCH), 1.83, 1.94 (s, 1:5, 3H, CH_3CN), 1.97, 1.99 (s, 5:1, 3H, CH_3CN), 2.57 (m, 2H, CH_2CO_2), 3.6 (m, 2H, CH_2N), 4.17 (q, $J = 7.1$ Hz, 2H, CH_2CH_3), 4.34, 4.55 (s, 1:10, 1H, *CH*), 6.6 (br s, 1H, *NH*), 8.31, 8.37 (s, 1:10, 1H, *HCO*). Mass Spectrum (EI): *m*/*z* (rel intensity) 316 ($M^{+\bullet}$, 7), 200 (32), 172 (100), 144 (80). Anal. ($C_{13}H_{24}N_2O_4S$) C,H,N.

D-Penicillamyl-*β*-alanine Hydrochloride (6d). A stirred solution of **7** (1.00 g, 3.16 mmol) in 40 mL of dioxane:2 N HCl (1:1) was heated under N_2 in a water batch at 85−90 °C. An aliquot sampled after 1.75 h for NMR analysis indicated about 8% starting material remaining. After 3.75 h, the reaction mixture was evaporated to dryness in vacuo, water was added, and the mixture was evaporated to dryness again. NMR analysis indicated about 3% of the formyl group was still uncleaved, although the ester moiety was hydrolyzed. The product was reheated in 15 mL of 1 N HCl for an additional 3.25 h under N_2 when NMR analysis indicated the reaction was complete. The solution was lyophilized to give 687 mg (79% yield) of **6d** as a hygroscopic solid, mp 71−75 °C. ^{1}H NMR (D_2O): δ 1.32 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 2.52 (t, $J = 6.4$ Hz, 2H, CH_2CO_2H), 3.31 (td, $J = 6.4$ Hz, $J = 14.0$ Hz, 1H, *CH*NCO), 3.50 (td, $J = 6.4$ Hz, $J = 14.0$ Hz, 1H, *CH*NCO), 3.78 (s, 1H, *CH*CS). Mass Spectrum (FAB): *m*/*z* 221 (MH^+). Anal. ($C_8H_{16}N_2O_3S{\cdot}HCl{\cdot}H_2O$) C,H,N.

***N*-(3-Formyl-2,2,5,5-tetramethylthiazolidinyl-4*S*-carbonyl)glycine *tert*-Butyl Ester (5a).** To an ice-cooled solution of **3** (5.75 g, 26.5 mmol) and Et_3N (7.50 mL, 5.45 g, 53.8 mmol) in CH_2Cl_2 (80 mL) was added isobutyl chloroformate (3.43 mL, 3.61 g, 26.5 mmol). The milky suspension was stirred for 1 h, and glycine *tert*-butyl ester hydrochloride (**4a**; 4.44 g, 26.5 mmol) was added. The reaction mixture was stirred with continued cooling for 30 min, then allowed to warm to room temperature overnight, and washed successively with 60-mL portions of 1 N HCl, 5% $NaHCO_3$, and H_2O. After drying over Na_2SO_4, the solution was evaporated to dryness in vacuo to give 8.38 g (97% yield) of **5a** as an amber oil which slowly crystallized. This product was dissolved in ether (100 mL) and diluted with hexane (100 mL), and the solution was evaporated to ca. 80 mL and cooled to give 5.19 g (60% yield) of **5a** as colorless needles, mp 112.5−113.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.49, 1.50 (s, 3:1, 9H, CH_3CO), 1.67, 1.72 (s, 7:1, 6H, CH_3CCH), 1.96, 1.98, 2.02 (s, 3:3:1, 6H, CH_3CN), 3.99 (m, 2H, CH_2), 4.4, 4.66 (s, 1:5, 1H, *CH*CON), 6.5 (br s, 1H, *NH*), 8.37, 8.41 (s, 1:6, 1H, *HCO*). Mass Spectrum (EI): *m*/*z* (rel intensity) 330

Scheme 1



($M^{+\bullet}$, 35), 257 (100), 200 (15), 172 (100), 144 (50). Anal. ($C_{15}H_{26}N_2O_4S$) C,H,N.

D-Penicillamylglycine Hydrochloride (6a). 5a (931 mg, 2.82 mmol) was hydrolyzed over 10 h using the same procedure for **6d** to give 587 mg of **6a** (77% yield) as a hygroscopic solid, mp 67−71 °C. 1H NMR (D2O): d 1.36 (s, 3H, CH3), 1.44 (s, 3H, CH3), 3.92 (s, 1H, CHCS) 3.92 (d, J = 17.8 Hz, 1H, CHNCO), 3.97 (d, J = 17.8 Hz, 1H, CHNCO). Mass Spectrum (FAB): m/z 207 (MH+). Anal. (C7H14N2O3S.HCl.1.5H2O) C,H,N.

***N*-(3-Formyl-2,2,5,5-tetramethylthiazolidinyl-4*S*-carbonyl)valine *tert*-Butyl Ester (5b). 5b** was prepared as for **5a** using 2.17 g (10.0 mmol) of **3** in 30 mL of CH_2Cl_2, Et_3N (3.0 mL, 2.78 g, 22.0 mmol), isobutyl chloroformate (1.30 mL, 1.37 g, 10.0 mmol), and **4b** (2.10 g, 10.0 mmol). After workup, the reaction mixture was evaporated to ca. 10 mL, diluted with hexane (20 mL), and evaporated to ca. 10 mL when a mass of colorless needles precipitated, mp 145.5−147 °C. A second crop of crystals was obtained by evaporation of the filtrate to ca. 1 mL and dilution with isopentane (5 mL). The total yield of **5b** was 2.11 g (57%). 1H NMR ($CDCl_3$): δ 0.94, 0.98 (d, 1:1, J = 6.8 Hz, J = 6.8 Hz, 6H, CH_3CH), 1.50, 1.51 (s, 2:1, 9H, CH_3-CO), 1.64, 1.67, 1.73 (s, 8:4:1, 6H, CH_3CCH), 1.97, 2.02 (s, 4:1, 6H, CH_3CN), 2.20 (m, 1H, $CH$$CH_3$), 4.4 (m, 1H, $CH$$CO_2$), 4.66 (s, 1H, CHCON), 6.6 (br d, 1H, *NH*), 8.36, 8.43 (s, 2:7, 1H, *HCO*). Mass Spectrum (EI): *m/z* (rel intensity) 372 ($M^{+\bullet}$, 65), 316 (100), 200 (100), 172 (100), 144 (100). Anal. ($C_{18}H_{32}N_2O_4S$) C,H,N.

D-Penicillamyl-L-valine Hydrochloride (6b). 5b (1.07 g, 2.87 mmol) was hydrolyzed for 6 h using the same procedure for **6d** to give, after lyophilization, 780 mg of **6b** (96% yield) as a fluffy solid, mp 263−265.5 °C dec. 1H NMR (D_2O): δ 0.84, 0.86 (d, 1:1, J = 6.8 Hz, J = 6.8 Hz, 6H, CH_3CH), 1.36 (s, 3H, CH_3CS), 1.43 (s, 3H, CH_3 CS), 2.10 (m, 1H, $CH$$CH_3$), 3.95 (s, 1H, CHCON), 4.15, 4.16 (d, 1:1, J = 5.7 Hz, J = 5.7 Hz, 1H, $CH$$CO_2$). Mass Spectrum (FAB): *m/z* 249 (MH^+). Anal. ($C_{10}H_{20}N_2O_3S\cdot HCl$) C,H,N.

***N*-(3-Formyl-2,2,5,5-tetramethylthiazolidinyl-4*S*-carbonyl)-α-aminoisobutyric Acid *tert*-Butyl Ester (5c).** 5c was prepared as for **5a** using 913 mg (4.20 mmol) of **3** in 50 mL of CH_2Cl_2, Et_3N (1.2 mL, 0.87 g, 8.6 mmol), isobutyl chloroformate (0.55 mL, 0.58 g, 4.2 mmol), and **4c·HCl·0.2H$_2$O** (811 mg, 4.07 mmol). After workup, the CH_2Cl_2 solution was evaporated to dryness, and the residue was purified by flash chromatography using EtOAc:hexane (1:4) as eluent to give 647 mg (61% yield) of a colorless oil as the byproduct **8**. 1H NMR ($CDCl_3$): δ 0.90 (d, J = 6.7 Hz, 6H, CH_3CH), 1.44 (s, 9H, CH_3CO), 1.49 (s, 6H, CH_3CN), 1.88 (m, 1H, $CH$$CH_3$), 3.80 (d, J = 6.7 Hz, 2H, CH_2CH), 6.4 (br s, 1H, *NH*). Anal. ($C_{13}H_{25}NO_4$) C,H,N.

Further elution with the same solvent mixture (2:3) gave 407 mg (28% yield) of **5c** as a colorless solid. Recrystallization from EtOAc:hexane (1:10) gave an analytical sample, mp 149.5−152 °C. 1H NMR ($CDCl_3$): δ 1.45, 1.48 (s, 5:1, 9H, CH_3-CO), 1.54, 1.55, 1.56, 1.62, 1.66 (s, 2:6:6:5:1, 12H, CH_3CCH and CH_3CCO_2), 1.91, 1.95, 1.97 (s, 3:3:1, 6H, CH_3CN), 4.24, 4.49 (s, 2:7, 1H, CHCON), 6.74 (br s, 1H, *NH*), 8.37, 8.41 (s, 1:6, 1H, *HCO*). Mass Spectrum (EI): *m/z* (rel intensity) 358 ($M^{+\bullet}$, 2), 200 (50), 172 (87), 144 (52). Anal. ($C_{17}H_{30}N_2O_4S$) C,H,N.

D-Penicillamyl-α-aminoisobutyric Acid Hydrochloride (6c). 5c (192 mg, 0.536 mmol) was hydrolyzed for 5 h using the same procedure for **6d** (except the water bath temperature was 65−70 °C) to give, after lyophilization, 150 mg of **6c** (100% yield) as a colorless solid, mp 145−148 °C. 1H NMR (D_2O): δ 1.37, 1.39, 1.448, 1.452 (s, 4:6:3:3, 12H, CH_3CS and CH_3CCO_2), 3.79, 3.80 (s, 1:1, 1H, CHCS). Mass Spectrum (FAB): *m/z* 235 (MH^+). Anal. ($C_9H_{18}N_2O_3S\cdot HCl$) C,H,N.

Sequestration of AcH. (a) In a cell-free system: AcH (40 nmol) was incubated without (control) and with the synthetic dipeptide or the amino acid (400 nmol) in a reaction mixture containing 4.0 mM dithiothreitol (DTT) and 40 mM potassium phosphate buffer (pH 7.4) in a total volume of 1.2 mL. The AcH remaining after a 30-min incubation period at 37 °C was quantified by headspace GC analysis.[13] DTT was included in the reaction mixture to maintain the thiols in their reduced state.

(b) In hepatocyte systems: Hepatocytes from male wistar rats were isolated and cultured under conditions previously described.[17] On culture day 2, ethanol and the test dipeptides were added to the culture medium to final concentrations of 20 and 1.0 mM, respectively. After 2 h, 1.0 mL of medium was removed and added to a semicarbazide/sodium azide mixture and frozen until assayed for AcH by gas chromatography.[17] AcH was measured before the additions and 2 h after incubation, and the values were compared against ethanol control without dipeptides. In experiments with cyanamide (to inhibit AIDH), the cyanamide was added with the ethanol to give a final concentration in the culture medium of 50 μM.

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