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2,5,5-Trimethylthiazolidine-4-carboxylic Acid, a D(−)-Penicillamine-Directed Pseudometabolite of Ethanol. Detoxication Mechanism for Acetaldehyde

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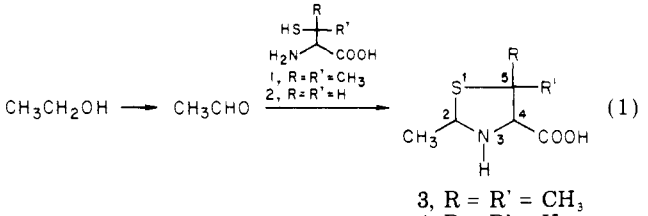
A directed detoxication mechanism for acetaldehyde (AcH) is described wherein ethanol-derived AcH, circulating in the blood of rats given ethanol-*1*-^{14}C and disulfiram or pargyline, was sequestered by condensation with administered D(−)-penicillamine (**1**). The product of this condensation, 2,5,5-trimethylthiazolidine-4-carboxylic acid (**3**), which was excreted in the urine without acetyl conjugation, was quantitatively determined by inverse isotope dilution measurements. Acetylation of the urine permitted the isolation of the corresponding *N*-acetyl derivative in crystalline form. The chirality of **3** was deduced by NMR analysis to be 72% 2*S*,4*S* and 28% 2*R*,4*S*. Although acetylation selectively acetylated the predominant isomer, this acetylated derivative was identical in all respects with a chemically synthesized product. This suggests that the in vivo condensation of AcH and **1** is not enzyme mediated.

The increasingly accepted hypothesis that acetaldehyde (AcH), the first metabolic product of ethanol, may be the causative agent in initiating the multitude of acute pharmacological and chronic pathophysiological effects of alcohol[1] suggested to us to seek methods to reduce its circulating blood levels. It was reasoned that the benefits of such lowered blood AcH might be protective for peripheral organs such as the heart and brain which contain only traces of alcohol dehydrogenase[2] and therefore are exposed only to *circulating* AcH—that which escaped hepatic metabolism.

We have succeeded in sequestering blood AcH generated from the metabolism of ethanol in rats by administration of the metabolically inert sulfhydrylamino acid, D(−)-penicillamine (**1**). This amino acid condensed with the ethanol-derived AcH in vivo to form the cyclic imino acid, 2,5,5-trimethylthiazolidine-4-carboxylic acid (**3**).[3] The elevation in blood AcH levels in disulfiram pretreated rats after ethanol administration, a consequence of aldehyde dehydrogenase inhibition by disulfiram, was sequestered by **1**[4] concomitant to a corresponding increase in the excretion of **3** in the urine, thus relegating the overall process (see eq 1) to a detoxication mechanism for AcH.



(1)

3, R = R' = CH_3
4, R = R' = H

A detailed proof of the structure of **3** and elucidation of the probable nonenzymatic nature of this in vivo condensation of AcH and **1** by reference to the chirality of synthetically produced **3** constitute the major thrust of this report. Additional in vivo experiments not previously reported are also described, showing the exclusivity of **1** in sequestering blood AcH compared to other sulfhydrylamino acid analogues and derivatives.

With respect to the stereochemical consideration alluded to, it is possible to infer a priori that since (a) a new chiral center at C-2 is generated in **3**, formed by the condensation reaction, and (b) the fixed chirality at the α position of D(−)-penicillamine (**1**)[5] should be unaffected during this process, any stereoselectivity imparted to the prochiral center, viz., to C-2, of the product isolated from rat urine (as in **3a** or **3b**, vide infra) could reflect an *enzymatic* mechanism for this condensation. On the other hand, lack of stereoselectivity at C-2 would relegate this to a nonenzymatic process. These considerations are somewhat nullified if steric constraints on **1** direct the incoming CH_3CHO to a sterically preferred orientation of the methyl group. Such asymmetric induction would impart to the C-2 carbon a preferred chirality which may not necessarily be enzyme directed.

Results

Detoxication Reaction for AcH. Our initial experiments were based on the expectation that administration of the natural sulfhydrylamino acid, L-cysteine (**2**), to ethanol-treated rats would direct its condensation with the ethanol metabolite, AcH, in vivo to the cyclic imino acid, 2-methylthiazolidine-4-carboxylic acid (**4**). Subsequent

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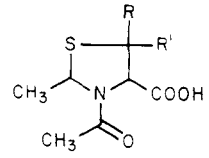
Table I. D(−)-Penicillamine-Directed Excretion of ^{14}C-Labeled 3 in Rats Given Ethanol-*1*-^{14}C

pretreatment	EtOH-*1*-^{14}C [3.0 g (65 mmol)/kg]	^{14}C-labeled 3 in 24-h urine,[a] mg	% of administered dose excreted as 3
D(−)-penicillamine [1.2 g (8.1 mmol)/kg]			
po[b]	po	12.3	0.4
po	po	29.2	1.2
ip	ip	14.6	0.5
po disulfiram	po	83.0	2.7
ip[c] disulfiram	ip[d]	145	7.6
ip pargyline	ip	110	3.3
ip pyrogallol	ip	26.4	1.1
L-cysteine, ip (10 mmol/kg)	ip	*e,f*	0
L-cysteine, ip (8.3 mmol/kg) disulfiram	ip	*e,f*	0
N-acetyl-L-cysteine, po (7.4 mmol/kg)	ip	*e,f*	0
N-acetyl-DL-penicillamine, po (8.0 mmol/kg)	ip	*f*	0

[a] Note that similar data presented in ref 3 are low by a constant factor of 4.72 due to a calculation error. [b] Simultaneously with ethanol-*1*-^{14}C; all others 1 h prior to ethanol-*1*-^{14}C. [c] In five divided doses of 2.0 mmol/kg at 0.25 h before and 0.75, 3, 5, and 7 h after ethanol-*1*-^{14}C. [d] 43.5 mmol/kg. [e] The isotope dilution carrier was 2-methylthiazolidine-4-carboxylic acid (4) isolated as its *N*-acetyl derivative, **5**. [f] The radioactivity of the isolated carrier was equal to the background radioactivity.

acetylation by an acetyl transferase to the *N*-acetyl derivative **6** would constitute a possible detoxication route



5, R = R' = CH_3
6, R = R' = H

for AcH, since **6** may be looked upon structurally as a cyclic mercapturic acid of AcH and therefore may be expected to be eliminated by the kidneys.

Administration of **2** 1 h prior to ethanol-*1*-^{14}C did not give rise to the urinary excretion of labeled **4** or its acetylated conjugate **6** as determined by inverse isotope dilution experiments (Table I). More conclusively, administration of **2** to a disulfiram-pretreated rat also given ethanol-*1*-^{14}C did not show the presence of labeled **4** or **6** in the urine. Likewise, administration of *N*-acetyl-L-cysteine did not lead to the excretion of **6**. Since administration of **4** itself did not give **6** in the urine of these rats,[3] the acetyl transferase that normally acetylates S-substituted cysteinyl conjugates of xenobiotic substances[6] apparently does not catalyze the acetylation of thiazolidine-4-carboxylic acid, a cyclic S-substituted cysteinyl derivative.

Trapping of ethanol-derived AcH in vivo was successfully accomplished by administration of the metabolically inert sulfhydrylamino acid, D(−)-penicillamine (**1**). **1** was effective whether given orally or by the intraperitoneal route or whether administered 1 h prior to or simultaneous with ethanol-*1*-^{14}C (Table I). The AcH was trapped as **3** (see eq 1) and excreted directly into the urine without acetyl conjugation as revealed by TLC.[3] However, isolation and quantitation by inverse isotope dilution assays were facilitated by acetylation of the ^{14}C-labeled **3** to the corresponding *N*-acetyl derivative **5**. Disulfiram given 24 h prior to ethanol-*1*-^{14}C caused increased excretion of ^{14}C-labeled **3** in the urine. The most effective treatment in this regard giving maximal excretion of **3** amounting to 7.6% of the administered dose of ethanol-*1*-^{14}C was by administration of **1** in multiple divided doses over 8 h. DL-Penicillamine at equimolar doses was toxic, presumably due to the presence of the L isomer. Again, administration of the preacetylated *N*-acetyl-DL-penicillamine followed by ethanol-*1*-^{14}C did *not* give labeled **5** in the urine (Table I).

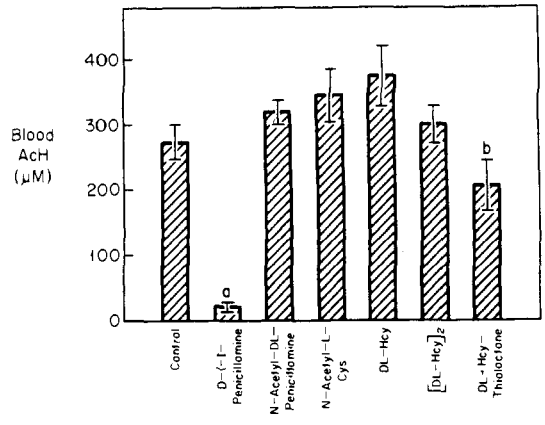


Figure 1. Effect of treatment with D(−)-penicillamine (**1**) and other sulfhydrylamino acids and derivatives on ethanol-derived blood AcH in rats given ethanol and disulfiram: (a) $p < 0.001$ vs. control; (b) $p > 0.10$. See Experimental Section for details.

Pargyline (*N*-methyl-*N*-propargylbenzylamine), a known MAO inhibitor which has been shown recently to be an aldehyde dehydrogenase inhibitor as well,[7] raises blood AcH levels in rodents after ethanol treatment in a like manner as disulfiram by virtue of the imposed block in the metabolism of AcH. Similarly, the antioxidant, pyrogallol, has been shown to elevate blood AcH levels,[8] presumably also by aldehyde dehydrogenase inhibition.[9] The urinary excretion of ^{14}C-labeled **3** after ethanol-*1*-^{14}C–**1** combinations was four times greater after pargyline pretreatment than after pyrogallol pretreatment (Table I).[10]

D(−)-Penicillamine (**1**) was the only effective agent for reducing in vivo the elevation in blood AcH levels due to the administration of disulfiram; L-cysteine (**2**) or L-cystine,[4] *N*-acetyl-L-cysteine, *N*-acetyl-DL-penicillamine, DL-homocysteine, DL-homocystine, and DL-homocysteine thiolactone were entirely without effect (Figure 1). Blood ethanol levels (not shown) were unaffected by these agents and were statistically not different from those of control animals (42 ± 2 mM).

Isolation of 3 (by Acetylation to 5) from Rat Urine. Since **3** was excreted without acetyl conjugation and is itself unstable, being dissociable to AcH and **1** at acidic pH values, it was necessary to collect the urine under alkaline conditions and to acetylate **3** to the more stable N-acetylated **5** for isolation. By pooling the urine from 6 to 10 rats, 50–200-mg quantities of pure, crystalline **5** were isolated by preparative TLC, the higher yields being obtained from disulfiram-pretreated rats. This permitted

Table II. Physicochemical Properties of Synthetic 3-Acetyl-2,5,5-trimethylthiazolidine-4-carboxylic Acid (5) Compared to the Product Isolated from Rat Urine

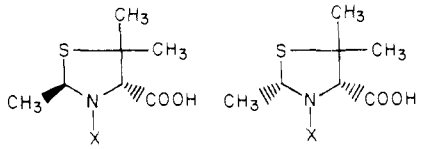
property	synthetic	product from rat urine
mp, °C	162-163	161-162
$[\alpha]^{24}_D$, deg (*c* 1.00, MeOH)	-39.5	-38.2, -39.6
Anal. ($C_9H_{15}NO_3S$)	C, H, N, S	C, H, N, S

	high-resolution mass spectra found *m/e* (rel intensity)	
formula (calcd mass)	synthetic	product from rat urine
$C_9H_{15}NO_3S$ (217.0753) [$M^{+\cdot}$]	217.0744 (1.3)	217.0774 (1.3)
$C_6H_{10}NO_2S$ (160.0413)	160.0430 (35.4)	160.0424 (30.8)
$C_6H_{12}NS$ (130.0671)	130.0686 (20.1)	130.0693 (18.3)
C_4H_7NOS (117.0229)	117.0250 (100)	117.0251 (100)
C_5H_8NS (114.0358)	114.0388 (17.1)	114.0378 (14.2)
C_4H_7S (87.0248)	87.0277 (8.1)	87.0266 (8.4)
C_4H_6NO (84.0449)	84.0458 (23.9)	84.0451 (22.7)

the direct comparison of the physicochemical properties of this biosynthetic urinary product with the chemically synthesized **5**.

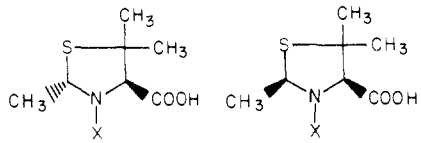
The IR, NMR, and chemical ionization mass spectra (CI-MS) of **5** isolated from rat urine, when compared to chemically synthesized **5** [Figures 2–4, respectively (see paragraph at end of paper regarding supplementary material)], were essentially superimposable. Their melting points, optical rotations, and high-resolution mass spectra are compared in Table II. These data established unequivocally that biosynthetic **5** was identical in all respects with the chemically synthesized product; nevertheless, they collectively did not yet establish the *absolute* chirality of **3** at C-2. The NMR spectrum of **5** did not resolve this question. Complications introduced by the puckering of the thiazolidine ring compounded by *E–Z* isomerism of the *N*-acetyl group[11] made difficult any stereochemical assignments by NMR data alone. Attempts to resolve this question by X-ray crystallographic analysis of biosynthetic **5** have not been successful to date.[12]

Chirality of 3 at C-2. Since chemically synthesized **3** gave the same N-acetylated derivative (**5**) as did **3** from rat urine (Table II), detailed stereochemical analysis of synthetic **3** should shed direct light on the chirality of **5**. Although asymmetric induction to a predominant *R* or *S* configuration at C-2 is possible during the formation of **3** (e.g., to **3a** or **3b**, respectively), we anticipated the formation of diastereomers and looked to effect their separation. However, chemically prepared **3** gave a homogeneous product when purified by sublimation giving nearly quantitative (94%) sublimation yields, and isolated second crops from reaction mixtures did not appear to be enriched with the other diastereomer. The melting points, optical rotations, and IR spectra of these second crops exhibited only minimal differences from the pure product, and their TLC mobilities were indistinguishable from each other. Despite this apparent homogeneity, the NMR spectrum of **3** showed it to be clearly a mixture, the C-4 methine protons of the diastereomers appearing at δ 4.07 and 3.87 in the approximate ratio of 1:3. Based on the observation that a C-2 methyl group in thiazolidine-4-carboxylic acids deshields the C-4 methine proton when cis to each other,[13]



2*R*,4*S* 2*S*,4*S*

3a, X = H **3b**, X = H
5a, X = $COCH_3$ **5b**, X = $COCH_3$



2*S*,4*R* 2*R*,4*R*

3c, X = H **3d**, X = H
5c, X = $COCH_3$ **5d**, X = $COCH_3$

the diastereomeric mixture represented by **3** must be 28% **3a**, where the stereochemistry is 2*R*,4*S*, and 72% **3b** with a 2*S*,4*S* stereochemistry. The stereoselective formation of the 2-methyl-4-carboxyl cis isomer **3b** corroborates the results of McMillan and Stoodley[13] who found a 1:4 distribution of isomers.

Acetylation of chemically prepared **3** above gave what appeared to be a single N-acetylated derivative with a single C-4 methine proton resonance at δ 4.57. However, the acetylation yields were not better than 64% to the pure product, and selective acetylation of only one of the diastereomeric forms, presumably the more abundant and thermodynamically more stable **3b**, could have given this result. By deduction, this N-acetylated derivative must be **5b** where the chirality is 2*S*,4*S*. This agrees with the exclusive cis stereoselectivity of the C-2 substituent and C-4 carboxyl group in the formation of 3-acetyl-2-(*p*-tolyl)thiazolidine-4-carboxylic acid as determined by X-ray crystallography.[14]

Condensation of AcH with L(+)-penicillamine gave a product with identical properties as **3**, obtained from AcH and D(−)-penicillamine (**1**), but having essentially equal specific rotation of opposite sign. NMR analysis revealed the same 1:3 mixture of diastereomers, predominated by the 2-methyl-4-carboxyl cis isomer, **3d**. Again, acetylation gave what appeared to be a single *N*-acetyl derivative, presumed to be **5d**, enantiomeric with that obtained from the D series. Finally, condensation of AcH with DL-penicillamine gave a mixture predicted from the above results, viz., 72% of the C-2,C-4 cis racemate (**3b** + **3d**) and 28% of the C-2,C-4 trans racemate (**3a** + **3c**). It follows that, during the reaction of AcH with penicillamine, the prochiral center at C-2 assumes a preferred orientation due to asymmetric induction by the chiral amino acid.

Discussion and Conclusions

The highly selective AcH sequestration in vivo by D-(−)-penicillamine (**1**) compared to the lack of efficacy of L-cysteine (**2**) is likely due to the metabolic inertness of the former[15] and the rapid catabolism of the latter.[16] In vitro, **2** condenses with AcH faster than does **1**.[17] The reason for the ineffectiveness of *N*-acetyl-L-cysteine, which can be hydrolyzed to **2** by a deacetylase in rat liver[18] and thus serve as a prodrug form of **2**, is not understood.

The absence of biochemical acetyl conjugation of **3** may be related to the cyclic and secondary nature of its amino group as well as its low basicity, the imino group of thiazolidine-4-carboxylic acid itself having a pK_a value of 6.21 compared to the amino group of *S*-ethylcysteine of 8.60.[19] It is noteworthy that thiazolidine-4-carboxylic acid,

a metabolic condensation product of DL-cystine and endogenous one-carbon fragments at the formaldehyde level of oxidation, is excreted in rat urine also without acetyl conjugation.[16a]

Based on the cumulative data presented above, albeit indirect, it can reasonably be concluded that the condensation of ethanol-derived AcH with D(−)-penicillamine (1) in vivo proceeds stereoselectively in an identical manner as the chemical reaction in the test tube. This suggests that the in vivo condensation is not necessarily a biochemical reaction requiring catalysis by enzymes; i.e., it may be nonenzymatic. Of interest is the observation that this D(−)-penicillamine-directed excretion of 3, a pseudometabolite of ethanol, is not restricted to rats. This compound was detected (as 5) in the urine of dogs given ethanol orally and 1 by infusion, as well as in the urine of two human volunteers who had consumed vodka and D-penicillamine.[20] Further studies on the protective effect of 1 on ethanol vis-à-vis acetaldehyde toxicity are in progress. The potential utility of 1 in sequestering other highly reactive, toxic intermediates produced in vivo during the metabolism of certain xenobiotic substances appears also to be worthy of investigation.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are corrected to reference standards. Optical rotations were measured in a Perkin-Elmer Model 141 polarimeter. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. The following spectrophotometers were used: IR, Beckman IR-10; NMR, Varian T-60A (Me_4Si internal standard); CI-MS (Dr. Roger Foltz, Battelle Columbus Laboratories, OH), AEI-MS-902 mass spectrometer equipped with an SRIC Model CRS-2 combined CI–EI ion source; and high-resolution MS, AEI-MS-30 (Department of Chemistry, University of Minnesota). A Packard Model 3375 liquid-scintillation spectrometer was used for radioactive counting. The penicillamines were all purchased from Aldrich Chemical Co., Milwaukee, WI.

2,5,5-Trimethylthiazolidine-4-carboxylic Acid (3). A. From D(−)-Penicillamine. D(−)-Penicillamine (11.95 g, 0.080 mol) was dissolved in 120 mL of H_2O at room temperature, and the mixture was clarified by filtration. The clear solution was cooled in an ice bath and 4.80 mL (3.76 g, 0.085 mol) of acetaldehyde was added at once with vigorous stirring. The reaction flask was stoppered to minimize the exposure of the contents to air. Within 15 min a voluminous white precipitate appeared. Stirring was continued for 1 h at ice-bath temperature and an additional 1.5 h at room temperature when a clear solution resulted. The solvent was evaporated to incipient dryness on a rotating evaporator, and the residual solids were dried further in a vacuum desiccator overnight. The crude product, mp 165.5–167.5 °C dec, sublimes >150 °C, was recrystallized from EtOAc by repeatedly digesting the solids in small portions of hot EtOAc and decanting the supernatant solution through a coarse, fritted glass filter (suction). The volume of the combined extracts was reduced to 300 mL, and crystallization was allowed to proceed at room temperature initially and then in the refrigerator overnight to give 11.05 g (78.8% yield) of product: colorless prisms; mp 166.5–168.5 °C dec (gas evolution), sublimes >150 °C; $[\alpha]^{23}_D$ +121.5° (*c* 1.00, MeOH), $[\alpha]^{26}_D$ +97.8° (*c* 1.0, pyridine) [reported[13] mp 149–150 °C; $[\alpha]_D$ +133° (py)]; NMR (pyridine-d_5) δ 4.80 (q, 1 H, *J* = 6 Hz, 2-H), 4.07 (s, 0.28 H, 4-H), 3.87 (s, 0.72 H, 4-H), 1.83 (s, 3 H, 5-CH_3), 1.52 (m, 6 H, 2,5-CH_3). Anal. ($C_7H_{13}NO_2S$) C, H, N, S. Evaporation of the mother liquor to dryness in vacuo gave 2.00 g of additional crude product which was recrystallized from EtOAc: colorless prisms; mp 165.5–166.5 °C dec (gas evolution) with sublimation >150 °C; 1.73 g (12.3%); $[\alpha]^{23}_D$ +116.2° (*c* 1.00, MeOH); total yield 12.78 g (91.2%).

A 1.50-g sample of the first crop above was purified by vacuum sublimation at 150–160 °C and 3 mmHg. All of the product sublimed within 2 h without leaving any residue to give 1.41 g of sublimate (94% yield, mechanical losses only): mp 167.5–168.5 °C dec (gas evolution), sublimes >150 °C; $[\alpha]^{23}_D$ +119.1° (*c* 1.00, MeOH); NMR (py-d_5) δ 4.77 (q, 1 H, *J* = 6 Hz, 2-H), 4.03 (s, 0.28 H, 4-H), 3.85 (s, 0.72 H, 4-H), 1.82 (s, 3 H, 5-CH_3), 1.50 (m, 6 H, 2,5-CH_3).

TLC on silica gel plates (Sil-G-25, UV 254, Machery-Nagel) using *n*-BuOH–HOAc–H_2O (50:11:25) or *n*-PrOH–H_2O (7:3) showed that the above three samples were not contaminated with starting D-penicillamine but had identical R_f values. The IR spectra of all these samples were identical: IR (KBr, cm^{-1}) 3250 (NH), 2470 (br, COOH dimer), 1730 (COOH), 1630 (NH deformation).

B. From L(+)-Penicillamine. Using L(+)-penicillamine in the above procedure gave, after recrystallization, 4.89 g (69.8% yield) of product: mp 166.5–167.5 °C dec (gas evolution) with sublimation >150 °C; $[\alpha]^{25}_D$ −117.5° (*c* 1.00, MeOH); IR (KBr, cm^{-1}) 3250 (NH), 2490 (br, COOH dimer), 1730 (COOH), 1630 (NH deformation); NMR (py-d_5) δ 4.83 (q, 1 H, *J* = 6 Hz, 2-H), 4.10 (s, 0.30 H, 4-H), 3.90 (s, 0.70 H, 4-H), 1.85 (s, 3 H, 5-CH_3), 1.53 (m, 6 H, 2.5-CH_3). Anal. ($C_7H_{13}NO_2S$) C, H, N, S. Crop 2: 1.21 g (17% yield); mp 163–166.5 °C; $[\alpha]^{24}_D$ −117.1° (*c* 1.00, MeOH). TLC gave the same results as in A.

C. Racemic. From DL-Penicillamine. Using DL-penicillamine in the above procedure gave, after recrystallization from ethanol, 71.9% yield of product: mp 186–188 °C dec with sublimation >170 °C; NMR (py-d_5) δ 4.80 (q, 1 H, *J* = 6 Hz, 2-H), 4.07 (s, 0.28 H, 4-H), 3.87 (s, 0.72 H, 4-H), 1.85 (s, 3 H, 5-CH_3), 1.53 (m, 6 H, 2,5-CH_3). Anal. ($C_7H_{13}NO_2S$) C, H, N, S. A second crop, mp 186–187 °C, was obtained in 11% yield by workup of the mother liquor above.

3-Acetyl-2(*S*),5,5-trimethylthiazolidine-4(*S*)-carboxylic Acid (5b). To a cooled, stirred solution of 3.50 g (0.020 mol) of 2,5,5-trimethylthiazolidine-4-carboxylic acid [3, from D(−)-penicillamine] in 50 mL of H_2O was added 3.18 g (0.030 mol) of anhydrous Na_2CO_3. When dissolution was nearly complete, 3.77 mL (4.08 g, 0.040 mol) of acetic anhydride was added dropwise over 10 min, and stirring was continued in the cold for 1 h. Acidification of the reaction mixture to pH 1 with concentrated HCl caused precipitation of the product which was collected, washed with H_2O, and dried in a vacuum desiccator over $CaCl_2$: colorless plates; mp 160–161 °C cor; 3.20 g (73.6% yield). This product was recrystallized from EtOAc [the suspended "fines" (NaCl) were removed by passing the hot solution through a bed of Celite analytical filter aid] to give 2.76 g (63.5% yield) of colorless, flat prisms: mp 162.5–163.5 °C, sublimes >140 °C; $[\alpha]^{24}_D$ −39.5° (*c* 1.00, MeOH); IR (KBr, cm^{-1}) 2690, 2575, 2480 (COOH dimer), 1735 (COOH), 1620 (amide CO), 1410 (CH_3), 1180 (C–O); CI-MS (isobutane) *m/e* (rel intensity) 218 (MH^+, 100), 200 (3), 176 (16), 117 (14); NMR (Me_2SO-d_6) δ 5.33 (q, 1 H, *J* = 6 Hz, 2-H), 4.57 (s, 1 H, 4-H), 2.10, 2.02 (2 s, 3 H, Ac), 1.63 (d, 3 H, *J* = 6 Hz, 2-CH_3), 1.50, 1.47 (2 s, 6 H, 5-CH_3) (see Figures 2–4, Supplementary Material). Anal. ($C_9H_{15}NO_3S$) C, H, N, S.

The acidic aqueous solution above was extracted twice with 50-mL portions of EtOAc, and the combined EtOAc extract was washed with 40 mL of saturated NaCl solution. After drying (Na_2SO_4), the solvent was evaporated to incipient dryness in vacuo, and toluene was added to the residue and then evaporated. Addition of toluene and evaporation were repeated three times to remove the acetic acid. The solid obtained thereby (0.50 g, mp 140–150 °C) was dissolved in the mother liquor from the recrystallization above, and the solvent was concentrated to give a second crop: 0.61 g (14.0% yield); mp 160.5–161.5 °C; $[\alpha]^{25}_D$ −35.0° (*c* 1.01, MeOH). TLC on silica gel GF (Analtech, Inc.) using BuOH–HOAc–H_2O (50:11:25) gave a single spot (I_2 chamber) for crop 1, R_f 0.8, but two spots, R_f 0.8 and 0.7, for crop 2, the second faint spot presumably the diastereomeric **5a** since it did not correspond to 1.

3-Acetyl-2(*R*),5,5-trimethylthiazolidine-4(*R*)-carboxylic Acid (5d). Acetylation of 3 obtained from L(+)-penicillamine using the above procedure gave **5d**. Crop 1 (68% yield): mp 163.5–164.5 °C, sublimes >150 °C; $[\alpha]^{25}_D$ +38.4° (*c* 1.06, MeOH). Crop 2 (10% yield): mp 162–163 °C; $[\alpha]^{25}_D$ +32.6° (*c* 1.00, MeOH). The NMR and IR spectra and TLC mobilities were identical with those of **5b**. Anal. ($C_9H_{15}NO_3S$) C, H, N, S.

Isolation of 3-Acetyl-2,5,5-trimethylthiazolidine-4-carboxylic Acid (5) from Rat Urine. Ten male Sprague–Dawley rats weighing 260–300 g and maintained without food for 24 h but with water ad libitum were treated with 2 mmol/kg of disulfiram (0.59 g/kg as a 50 mg/mL solution in 5% gum acacia)

by oral intubation. Nineteen hours later the rats were given 1.2 g (8.1 mmol)/kg of D(−)-penicillamine (as a 100 mg/mL solution in water) intraperitoneally (ip), followed 1 h later by 3.0 g (6.5 mmol)/kg of ethanol (200 mg/mL in isotonic saline) ip. Each rat was individually caged and had access to food and water for the remainder of the experiment. The 24-h urine samples were collected over Na_2CO_3 (1.0 g per rat) and then pooled, and the urine was acetylated by cooling in an ice bath and adding 5.9 g of Ac_2O dropwise over 10 min with stirring. Stirring was continued in the cold for 0.5 h, and the reaction mixture was acidified to pH 1 with concentrated HCl. After saturation with NaCl, the mixture was extracted three times with 60-mL portions of EtOAc. (The first two extractions required centrifugation to break up the emulsions which had formed.) The combined EtOAc extract was dried over anhydrous Na_2SO_4, decolorized with activated carbon, and concentrated to a volume of less than 5 mL. This material was purified by chromatography on two 20 × 20 cm preparative TLC plates (Analtech silica gel GF, 1000 μm) using $CHCl_3$–HOAc (4:1) as the solvent. The bands corresponding to the desired product were removed and extracted with EtOAc, and the concentrated extract was rechromatographed on preparative TLC plates and processed as above. Evaporation of the solvent gave a crystalline residue which was dried in vacuo overnight. Recrystallization twice from EtOAc yielded 0.18 g of **5**: mp 161–162 °C; IR (KBr, cm^{-1}) 2690, 2575, 2480 (COOH dimer), 1735 (COOH), 1620 (amide CO), 1410 (CH_3), 1180 (C–O); CI-MS (isobutane) *m/e* (rel intensity) 218 (MH^+, 100), 200 (3), 176 (16), 117 (14); NMR (Me_2SO-d_6) δ 5.35 (q, 1 H, J = 6 Hz, 2-H), 4.57 (s, 1 H, 4-H), 2.12, 2.02 (2 s, 3 H, Ac), 1.64 (d, 3 H, J = 6 Hz, 2-CH_3), 1.50, 1.47 (2 s, 6 H, 5-CH_3) (see Figures 2–4, Supplementary Material).

Inverse Isotope Dilution Measurements of 3 Excreted in Rat Urine. In typical experiments, individual male Sprague-Dawley rats (Madison, WI) weighing 190–280 g were pretreated (or not treated) with one of the aldehyde dehydrogenase inhibitors, disulfiram (2.0 mmol/kg po, 24 h prior to ethanol-*1*-^{14}C), pargyline (0.63 mmol/kg ip, 2 h before ethanol-*1*-^{14}C), or pyrogallol (2.0 mmol/kg ip, 1 h before ethanol-*1*-^{14}C). D(−)-Penicillamine or one of the other sulfhydrylamino acids or derivatives (doses and mode of administration as indicated in Table I) was administered 1 h before ethanol-*1*-^{14}C (65 mmol/kg as a 200 mg/mL solution in isotonic saline; 0.8–1.4 μCi per rat). The rat was placed in a glass metabolism cage (Coleman–Delmar) which permitted the separate collection of urine, feces, and expired $^{14}CO_2$, the latter in two consecutive CO_2 traps (100 mL of 10 M NaOH and 250 mL of 10 M NaOH). Air scrubbed for CO_2–H_2O (Ascarite and Drierite tower) was pulled through the cage at rates of 180–380 mL/min. The excreted urine was periodically drained into an Erlenmeyer flask containing 1.0 g of solid Na_2CO_3. At 12 h, the primary $^{14}CO_2$ trap was replaced with a new trap containing fresh 10 M NaOH.

After 24 h, the rat was removed and the cage rinsed with H_2O. The combined cage washings and urine were gravity filtered to remove food, hair, and other solid debris, and 400 mg of unlabeled carrier (**3** or, as the case may be, **4**) was added to the filtrate. The volume was adjusted to 100.0 mL with H_2O and two 1.0-mL aliquots were taken, and the radioactivity was counted in 10 mL of scintillation cocktail consisting of 150 parts of Triton X-100 and 330 parts of scintillation solution (4.0 g of PPO and 0.3 g of POPOP dissolved in 1 L of toluene).

The diluted urine containing carrier was acetylated with 5 mL of Ac_2O, and the acetylated product was purified and isolated by preparative TLC essentially as described above for **5** (from rat urine). The isolated carrier (usually 0.3–0.4 g) was recrystallized from EtOAc to constant specific radioactivity. Samples (25 mg) were dissolved in 5 mL of 50% aqueous ethanol, and 1.0-mL aliquots were counted in the scintillation cocktail described above. The amount of labeled **3** excreted in 24-h urine was calculated. These results are presented in Table I.

A major function (70–90%) of the administered dose of ethanol-*1*-^{14}C was excreted as $^{14}CO_2$ in 24 h, as determined by assaying[21] the $^{14}CO_2$ content of the NaOH traps.

Sequestration of Blood AcH in Vivo. Groups of male rats weighing 220–310 g with a minimum of five animals per group were used. The animals were fasted for 24 h prior to receiving a single dose of disulfiram (2.0 mmol/kg po, as a 60 mg/mL suspension in 5% gum acacia). The AcH sequestering agent (8.0 mmol/kg ip) or vehicle alone (controls) was administered 24 h after disulfiram treatment. This was followed by ethanol (43.5 mmol/kg as a 20% w/v aqueous solution, ip) 1 h later. Blood was collected by open-chest cardiac puncture 1 h after ethanol administration. Blood AcH and ethanol were measured using a head space gas chromatographic procedure as previously described.[4] The results are shown in Figure 1.

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Supplementary Material Available: IR spectra (Figure 2), NMR spectra (Figure 3), and CI-MS (Figure 4) of 3-acetyl-2,5,5-trimethylthiazolidine-4-carboxylic acid isolated from rat urine compared against a chemically synthesized product (3 pages). Ordering information is given on any current masthead page.

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Biologically Active Derivatives of Angiotensin for Labeling Cellular Receptors

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The synthesis and potencies of several amino-terminal derivatives of angiotensin II to be used for the labeling of its receptors on vascular smooth muscle cells are reported here. The relative affinities on rabbit aortic strips of the compounds prepared, based on a potency of 100% for [Asp[1],Ile[5]]angiotensin II (**4**), are N^{α}-(4-formylbenzoyl)-[Asn[1],Val[5]]angiotensin II (**5**) 8%, N^{α}-(*N*-fluoresceinthiocarbamyl)-[Asp[1],Ile[5]]angiotensin II (**6**) 1%, N^{α}-(*N*-rhodaminethiocarbamyl)-[Asp[1],Ile[5]]angiotensin II (**7**) 4%, [2-methoxy-2,4-diphenyl-3(2*H*)-furanone]-[Asp[1],Ile[5]]-angiotensin II (**8**) 3%, and N^{α}-[(2-nitro-5-azidobenzoyl)norleucyl]-[Asp[1],Ile[5]]angiotensin II (**9**) 0.5%. Free angiotensin II was not detectable in peptides **5–9**. With the exception of **6** and **7** all peptides were of high purity by paper electrophoresis and thin-layer chromotography. Therefore the biological activities found for both **6** and **7** have the limitations that they represent the activity of a mixture. The peptides were not tested for antagonist activity.

Two classes of derivatives of angiotensin have been prepared for the study of its vascular receptor sites: fluorescent derivatives for light microscopic visualization of receptors (**6–8**) and reactive derivatives for the covalent labeling of receptors (**5** and **9**).

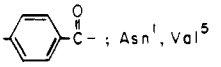
Fluorescent derivatives of small peptide hormones have rarely been prepared and characterized. However, N^{α}-(*N*-fluoresceinthiocarbamyl)tetragastrin[1] and fluorescamine labeled [Asp[1],Ile[5]]angiotensin II[2] have been prepared by others and have been found to have high biological activity. We describe here the fluorescent derivatives of angiotensin with fluorescein isothiocyanate (**6**), tetramethylrhodamine isothiocyanate (**7**), and 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF, **8**). These three compounds were prepared in order to have a range of fluorescence emission wavelengths for biological studies with vascular smooth muscle cells. Emission maxima for proteins derivatized with these fluorophores have been previously reported: fluorescein isothiocyanate–protein conjugates,[3] λ_{max} >520 nm, tetramethylrhodamine–protein conjugates,[3] λ_{max} >595 nm, and MDPF–γ-globulin conjugates,[4] λ_{max} 480 nm.

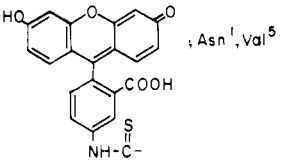
Several examples of chemically reactive peptide hormones are known. Chlorambucil-angiotensin II and chlorambucil-[des-Asp[1],Val[5]]angiotensin II have been shown to be irreversible, noncompetitive inhibitors of angiotensin II on guinea pig ileum and rat uterus,[5] whereas chlorambucil-[des-Asp[1],Val[8]]angiotensin I had no irreversible effects on guinea pig ileum, rat uterus, rabbit aorta, or rat blood pressure.[6] Chlorambucil-bradykinin had some irreversible effects on guinea pig ileum in vitro and on pulmonary kininases in vivo,[7] while (bromoacetyl)bradykinins had no irreversible effects on rat uterus in vitro or on rat blood pressure.[8] (Bromoacetyl)oxytocin was found to be an irreversible inhibitor of neurohypophyseal hormone stimulated adenylate cyclase in toad bladder, but only at 0.1 mM, where oxytocin itself was also an inhibitor.[9] Maleoyl derivatives of oxytocin had no irreversible effects on toad bladder or rat uterus in vitro.[10] Using a different approach, photolyzable aryl azide derivatives of peptide hormones have been described which are capable of covalent bond formation to any amino acid residues present in the hormone receptor site. Photolyzable insulin derivatives with high biological potency have been reported but were not tested for irreversible biological effects.[11] A photolyzable aryl azide derivative of a cholecystokinin fragment has been shown to be an irreversible agonist in stimulating discharge of exportable proteins from guinea pig exocrine pancreas in vitro.[12] Finally, a similar pho-

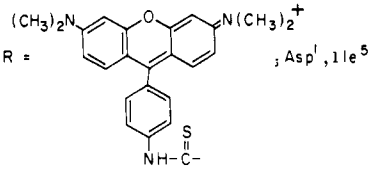
R-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

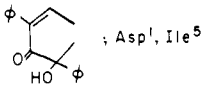
1, R = H; Asn[1], Val[5]

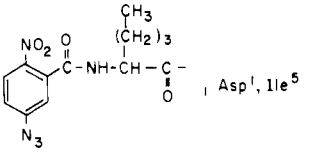
4, R = H; Asp[1], Ile[5]

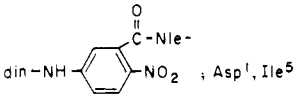
5, R =  ; Asn[1], Val[5]

6, R =  ; Asn[1], Val[5]

7, R =  ; Asp[1], Ile[5]

8, R =  ; Asp[1], Ile[5]

9, R =  , Asp[1], Ile[5]

10, R = avidin-NH-  ; Asp[1], Ile[5]

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