temperature of the sample could therefore at most be increased ca. 0.3 °C by the noise decoupling. In this way, the calibration of the chemical-shift thermometer was improved (CH_3OH in a mixture of $CHCl_2F$-$CDClF_2$, 1:1, v/v)[1d,2b] at one temperature. Most of the spin-transfer experiments were performed at ca. −128 °C, and, therefore, the above careful temperature calibration at one temperature of the shift thermometer automatically gave accurate temperature estimates over a large interval around −119 °C.

The maximal error estimates shown in Table II are conservative, since they have been obtained through propagation of maximal errors.

The higher temperatures used were not calibrated in the way described above and therefore the estimated error limits are much larger. The separation of the CH_3 and OH proton bands of the shift thermometer was measured before and after each NMR experiment. Since these separations were usually found to vary <~0.1 Hz (i.e., <0.1 °C) from the average value, the temperature stability during the kinetic experiments was concluded to be within ±0.1 °C.

All spin-transfer experiments showed reproducibility within the limits shown in Table II. If there is any temperature gradient over the sample part of the NMR tube, it cannot be subtracted from the above measurements, but can probably be neglected as if diffusion of molecules is significant (which is unlikely at this low temperature); the number of molecules of a certain kind diffusing into the central part used for the observation is probably similar to that diffusing away.

Acknowledgments. We wish to thank Dr. Ulf Obenius for NMR technical assistance and the Swedish Natural Science Research Council for support.

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Elucidation of the Stereochemistry of the Carboxypeptidase A Catalyzed Enolization of 2-Benzyl-3-*p*-methoxybenzoylpropionate, a Ketone Substrate

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Abstract: Carboxypeptidase A_γ catalyzes hydrogen-deuterium exchange with retention of configuration at the activated methylene group of (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid, (−)-**1**, a ketonic analogue of the enzyme's ester and peptide substrates. However, (+)-**1** does not undergo the corresponding exchange process even though it is bound to the enzyme slightly more tightly than the − isomer. ^1H NMR measurements at 270 MHz show that the signals for the diastereotopic protons on the activated methylene group of **1** appear separately at 2.98 (H_a) and 3.33 ppm (H_b) and that exchange occurs only at the H_a position of (−)-**1**. Degradation of the enantiomers of **1** by Baeyer–Villiger oxidation and subsequent hydrolysis to give the corresponding isomers of 2-benzylsuccinic acid showed that (−)-**1** has the *R* configuration at the chiral methine group. *cis*- and *trans*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones containing hydrogen and/or deuterium at the 3-methylene position were prepared by sodium borohydride reduction of **1** and various of its deuterated forms, followed by cyclization using dicyclohexylcarbodiimide. Comparison of the ^1H NMR spectra of the lactones with those of the *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones produced by the cyclization of **1** and **1**-d_2 (substituted with deuterium at the 3 position) with acetic anhydride, followed by reduction with hydrogen over platinum oxide, showed that the H_a proton on the activated methylene group is in the *pro-R* configuration. The stereochemical results obtained are consistent with the hypothesis that (−)-**1** binds in a manner similar to that which has already been postulated for reactive peptide and ester substrates and that the γ-carboxylate moiety of Glu-270 is the functional group in the enzyme which abstracts the H_a hydrogen from the 3 position of the ketone substrate.

Potent nucleophiles are known to be present at the active sites of many enzymes involved in acyl- and phosphoryl-transfer processes. While kinetic studies and chemical modification experiments have been powerful mechanistic tools, often the multiplicity of reaction intermediates, the possibility of a change in what is the rate-determining step as the pH is changed, and other factors can combine to make it exceedingly difficult, even with these techniques, to discern clearly the catalytic behavior of the crucial enzyme-bound nucleophiles. Recently, we undertook experiments to test whether a relatively

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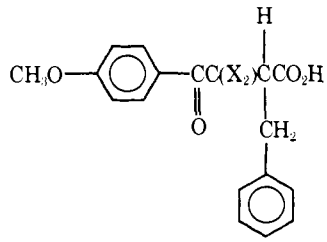
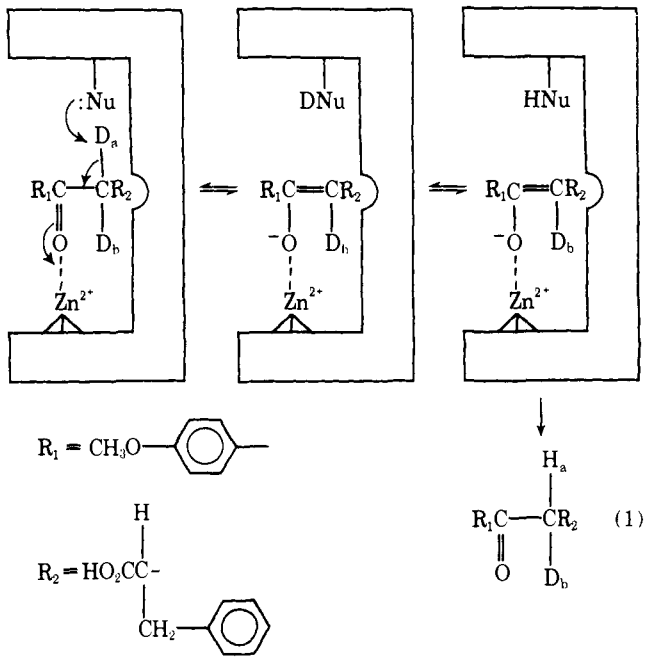
simple enolization reaction in which the problem of multiple intermediates would not occur could be used to probe the catalytic activity of the carboxylate group of the essential Glu-270 residue in the active site of carboxypeptidase A, a hydrolytic enzyme. In a preliminary communication,[1] we reported our discovery that carboxypeptidase A_γ catalyzes stereospecific hydrogen-deuterium exchange at the activated methylene group of (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid ((−)-**1**), a ketonic analogue of ester and peptide substrates of the enzyme. Exchange was not seen for (+)-**1**, although binding of this isomer to the enzyme was observed. Peaks for the diastereotopic protons on the methylene group of **1** were observed to appear separately at 2.98 (H_a) and 3.33 ppm (H_b) in ^1H NMR measurements at 270 MHz. Since exchange occurring only at the H_a position of (−)-**1** was observed at neutral pH, it was concluded that this reaction occurs with retention of configuration.



1a, X = H
b, X = D

On the basis of the structural and mechanistic information available on carboxypeptidase A the scheme shown in eq 1 in



which the active site zinc ion holds the carbonyl group of the ketonic substrate in place while a nucleophile Nu abstracts the hydrogen (deuterium) from the H_a position provides a reasonable hypothesis to account for the carboxypeptidase A_γ catalyzed enolization of (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid ((−)-**1**). Furthermore, if the binding of the ketone (−)-**1** occurs in a manner similar to that postulated for reactive peptide and ester substrates,[2,3] and if the nucleophile Nu is the γ-carboxylate of Glu-270, then this will have specific implications for the stereochemistry at the chiral methine position and at the prochiral activated methylene position of (−)-**1**. We now report the results of experiments that we have performed to establish the absolute configuration at the asymmetric 2-methine position of (−)-**1** and at the *pro*-chiral 3-methylene position where the hydrogen-deuterium exchange reaction takes place.

Experimental Section

^1H NMR spectra were recorded at 270 MHz on a Bruker HS-270 spectrometer equipped with a Nicolet data processor system. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter, employing a 10-cm pathlength cell. Alkaline hydrolysis at constant pH was performed using a Radiometer pH stat (Titrator 11, Autoburette ABU 11, Titrigraph SBR2c, Assembly TTA31).

The following chemicals were purchased commercially and used without further purification: acetic anhydride (Mallinckrodt, reagent grade), *m*-chloroperbenzoic acid (Aldrich), dicyclohexylcarbodiimide (Aldrich), and sodium borohydride (Sigma). Other commercially purchased chemicals were purified by distillation before use: dimethylformamide and pyridine were distilled over barium oxide at atmospheric pressure; chloroform was washed with water, concentrated sulfuric acid, water, and then distilled from phosphorus pentoxide. (+)- and (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid (**1a**) and (+)- and (−)-2-benzyl-3-*p*-methoxybenzoylpropionic acid-*3,3-d*$_2$ (**1b**) were prepared and resolved as described previously.[1] Carboxypeptidase A (γ-isozyme) was purchased from Worthington Biochemical Corp. (Lot No. 1681 COA 37 K678). The enzyme stock solution was prepared as described by Kaiser and Carson.[4]

Baeyer-Villiger Oxidation of (−)-1a. A solution of (−)-**1a** (81 mg, 0.27 mmol) and *m*-chloroperbenzoic acid (182 mg, 0.90 mmol) in chloroform (2.5 mL) was kept at room temperature for 3 days. After evaporation of the solvent under vacuum at room temperature, the slightly yellowish residue was dissolved in 2 mL of ethyl acetate and transferred to a 10-mL vessel to be used with the Radiometer pH stat, and the solvent was removed by flushing with nitrogen. To the vessel 5 mL of 0.1 N sodium hydroxide was added with stirring. Subsequently, 1 N sodium hydroxide was added dropwise to adjust the solution to approximately pH 11.5, and, using the pH stat and 0.1 N sodium hydroxide titrant, the solution was maintained at pH 12 for 2 h until all of the residue was dissolved. After removal of the vessel from the pH stat, concentrated hydrochloric acid was added until the solution was below pH 3, and then the white precipitate formed was immediately removed by filtration. The precipitate was washed with dilute hydrochloric acid (5 mL). The combined filtrate (15 mL) was extracted with ethyl acetate (2 × 30 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. After recrystallization twice from ethyl acetate–petroleum ether, (+)-2-benzylsuccinic acid ((+)-**2**) was obtained: mp 157.5–160 °C (lit.[5] mp 159–161 °C); 26.0 mg, 0.12 mmol, yield 46%. The NMR spectrum (in acetone-d_6) was identical with that of the authentic compound. The optical rotation measured is listed in Table I. In a similar manner, from (+)-**1a** (81 mg, 0.27 mol) and *m*-chloroperbenzoic acid (180 mg, 0.89 mmol) ((−)-**2**), was obtained: mp 158–160 °C; 32 mg, 0.15 mmol, yield 57%. The optical rotation measured is shown in Table I.

Conversion of 1a to *cis*- and *trans*-2-Benzyl-4-*p*-methoxyphenyl-γ-butyrolactones (3a and 4a). A mixture of (+)-**1a** (151 mg, 0.51 mmol) and sodium borohydride (54 mg, 1.43 mmol) was dissolved in dimethylformamide (5 mL) and allowed to stand at room temperature for 3 h. Dilute hydrochloric acid (3 mL) was added to destroy excess sodium borohydride. After evolution of hydrogen had ceased, the reaction mixture was poured into water (20 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed with saturated aqueous sodium chloride (50 mL), dried over anhydrous sodium sulfate, and evaporated under vaccum. The residue and dicyclohexylcarbodiimide (200 mg, 0.97 mmol) were dissolved in pyridine (5 mL) and stirred overnight. Then the pyridine solution was poured into dilute hydrochloric acid and stirred with ethyl acetate for 1 h. After the white precipitate was removed by filtration, the organic layer was separated, washed with saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. The ethyl acetate solution was concentrated at room temperature under vacuum to a volume of 2 mL. Then the concentrated reaction mixture was applied to a silica gel column (19-mm diameter, 20-cm length) and eluted with benzene. From the intermediate fractions, a mixture of *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactone (**3a**) and *trans*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactone (**4a**, 112 mg) was obtained. From a comparison of the signal intensities at 5.20 (4-methine proton of cis isomer, **3a**; see below) and 5.33 ppm (4-methine proton of trans

isomer, **4a**; see below) the ratio of **3a** and **4a** was estimated to be 4.1. Separation of **3a** and **4a** was performed by means of preparative thin layer chromatography on silica gel. The mixture (28 mg) was applied to a silica gel TLC plate (0.25-mm-layer thickness, 20 cm × 20 cm) and eluted using ether–petroleum ether (1:1); **3a** (13 mg, R_f 0.20) and **4a** (3 mg, R_f 0.28) were isolated. **3a**: NMR ($CDCl_3$) δ 1.92 (dd, 1 H, J = 12.1, 22.6 Hz), 2.55 (m, 1 H), 2.80 (dd, 1 H, J = 9.5, 13.8 Hz), 3.04 (m, 1 H), 3.34 (dd, 1 H, J = 4.1, 13.8 Hz), 3.80 (s, 3H), 5.20 (dd, 1 H, J = 6.0, 11.1 Hz), 6.87 (d, 2 H, J = 8.7), 7.1~7.36 (m, 7 H); IR (KBr) 1772 cm^{-1} (C=O). Anal. Calcd for $C_{18}H_{18}O_3$: mol wt, 282.1255. Found: 282.1258. **4a**: NMR ($CDCl_3$) δ 2.20 (m, 1 H), 2.41 (m, 1 H), 2.86 (dd, 1 H, J = 8.5, 13.6 Hz), 3.00 (m, 1 H), 3.25 (dd, 1 H, J = 4.0, 13.6 Hz), 3.80 (s, 3 H), 5.33 (dd, 1 H, J = 5.5, 7.7 Hz), 6.87 (d, 2 H, J = 8.5 Hz), 7.14–7.40 (m, 7 H); IR (KBr) 1767 cm^{-1} (C=O). Anal. Calcd for $C_{18}H_{18}O_3$: mol wt, 282.1255. Found: 282.1239.

Conversion of 1b to *cis*- and *trans*-2-Benzyl-4-*p*-methoxyphenyl-γ-butyrolactones-3,3-d_2 (3b and 4b). Starting from **1b** (126 mg, 0.42 mmol) and sodium borohydride (60 mg, 1.59 mmol), a mixture (100 mg) of *cis*- and *trans*-2-benzyl-γ-butyrolactones-3,3-d_2 (**3b** and **4b**), respectively, was obtained. The isolation of pure **3b** and **4b** was carried out using preparative TLC (silica gel) with ether–petroleum ether (1:1) as the eluant. **3b**: NMR ($CDCl_3$) δ 2.80 (dd, 1 H, J = 9.5, 13.8 Hz), 3.04 (m, 1 H), 3.34 (dd, 1 H, J = 4.1, 13.8 Hz), 3.80 (s, 3 H), 5.20 (br s, 1 H), 6.87 (d, 2 H, J = 8.7 Hz), 7.1–7.4 (m, 7 H). **4b**: NMR ($CDCl_3$) δ 2.86 (dd, 1 H, J = 8.5, 13.6 Hz), 3.00 (m, 1 H), 3.25 (dd, 1 H, J = 4.0, 13.6 Hz), 3.80 (s, 3 H), 5.33 (br s, 1 H), 6.87 (d, 2 H, J = 8.5 Hz), 7.14–7.80 (m, 7 H).

Deuterium–Hydrogen Exchange of (−)-1b Catalyzed by Carboxypeptidase A. A solution of (−)-**1b** (17.2 mg) in 6.2 mL of 0.05 M Tris HCl buffer (pH 7.5, 0.5 M NaCl) and 7.0 mL of CPA stock solution (1.62×10^{-4} M, 0.05 M Tris HCl buffer, pH 7.5, 0.5 M NaCl) were mixed at room temperature and kept at 25 °C for 70 h. After 0.5 mL of hydrochloric acid (5 N) was added, the reaction mixture was extracted with ethyl acetate (2 × 50 mL). The combined ethyl acetate solution was washed with saturated aqueous sodium chloride (50 mL) and dried over anhydrous sodium sulfate. The solvent was removed by evaporation under vacuum. The signal intensities observed in the ^{1}H NMR spectrum of the sample isolated from the exchange process indicated that 0.74 atom H and <0.05 atom H were introduced at the H_a (2.98) and H_b (3.33 ppm) positions, respectively.

Conversion of a Sample from Enzyme-Catalyzed Exchange of (−)-1b to the Corresponding γ-Butyrolactones. The procedure described for the conversion of **1a** and **1b** to the corresponding *cis*- and *trans*-2-benzyl-4-*p*-methoxybenzyl-γ-butyrolactones was employed for the exchanged material. A 15-mg sample was reduced with sodium borohydride (37 mg, 0.98 mmol) in dimethylformamide (5 mL). Lactonization of the reduced material was carried out using dicyclohexylcarbodiimide (100 mg, 0.48 mmol) in pyridine (3 mL). The pure *cis*- and *trans*-γ-butyrolactones were isolated by preparative TLC (silica gel) with ether–petroleum ether (1:1) as the eluant. The ^{1}H NMR spectra of the lactones obtained are shown in Figures 1 and 2.

Synthesis of 2-Benzyl-4-*p*-methoxyphenylbut-2-en-4-olide (5a). A solution of **1a** (1.0 g, 3.4 mmol) in acetic anhydride (200 mL) was refluxed for 3 h. The acetic anhydride was removed under vacuum, and the residue was chromatographed over silica gel (19-mm diameter column, 20-cm length) using benzene as the eluant. From the intermediate fractions **5a** (0.50 g, 1.8 mmol) was obtained: NMR ($CDCl_3$) δ 3.66 (s, 2 H), 3.78 (s, 3 H), 5.81 (d, 1 H, J = 2 Hz), 6.85–6.90 (m, 3 H), 7.12 (d, 2 H, J = 8.6 Hz), 7.2–7.5 (m, 5 H). Anal. Calcd for $C_{18}H_{16}O_3$: mol wt, 280.1099. Found: 280.1096.

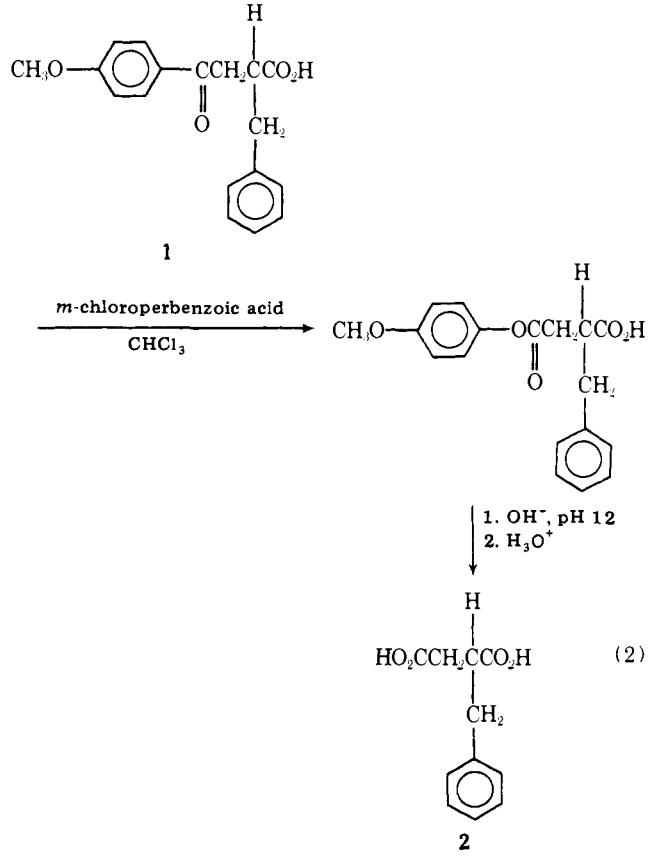
Catalytic Hydrogenation of 5a. A mixture of platinum oxide (40 mg) and **5a** (0.50 g) in ethyl acetate (10 mL) was shaken under atmospheric pressure of hydrogen until 44 mL of hydrogen was absorbed. The reaction mixture obtained was filtered, and the solvent evaporated. The residue was dissolved in boiling ether–petroleum ether (1:1, 30 mL). The insoluble material remaining was removed by filtration and the solvent was then evaporated. Recrystallization from ether–petroleum ether (1:3) gave **3a** (200 mg). The NMR spectrum of this material was identical with the one obtained in the preparation of **3a** above. Anal. Calcd for $C_{18}H_{18}O_3$: C, 7.57; H, 6.43; O, 17.00. Found: C, 76.64; H, 6.60; O, 17.04.

To eliminate the possibility that the *trans*-γ-butyrolactone **4a** is lost during the isolation or recrystallization steps, the NMR spectrum of the reaction mixture was measured before the completion of hydrogenation. The only compounds found to be present were the *cis*-γ-butyrolactone **3a** and the starting unsaturated lactone **5a**.

Synthesis of *cis*-2-Benzyl-4-*p*-methoxyphenyl-γ-butyrolactone-3-*d* (3c). The procedure employed corresponded to that described in the paragraph above. A solution of **1b** (1.0 g, 3.3 mmol) in acetic anhydride (20 mL) was refluxed for 3 h. By chromatography over silica gel 2-benzyl-4-*p*-methoxyphenylbut-2-en-4-olide-3-*d* (**5b**, 0.58 g) was isolated. Catalytic hydrogenation of **5b** was carried out over platinum oxide (49 mg) in ethyl acetate (10 mL) until 75 mL of hydrogen was absorbed. Recrystallization from ether–petroleum ether (1:3) gave **3c** (262 mg). The NMR spectrum corresponded to that of **3a** except for the following differences: 1.92 (dd, <0.1 H), 2.55 (br t, 1 H, J = 6.0 Hz), 3.04 (m, 1 H), 5.20 (d, 1 H, J = 5.3 Hz).

Results and Discussion

In our previous communication we observed the occurrence of hydrogen–deuterium exchange at the activated methylene group only in the case of (−)-**1** and not with (+)-**1**. On the basis of the hypothesis that it is the enantiomer of **1** having the same configuration at the methine position as that of reactive peptide or ester substrates which is incorporated properly into the active site so that the methylene group at the 3 position is within reach of the catalytic groups of the enzyme, we tentatively assigned the *R* configuration to (−)-**1** and the *S* configuration to (+)-**1**. To establish the absolute configuration of (−)-**1** and (+)-**1**, determining the validity of our assignment, in the present work we undertook the degradation of **1** to 2-benzylsuccinic acid (**2**) for which the absolute configuration of the enantiomers had already been established. The sequence of reactions carried out is illustrated in eq 2. In the first step the



(2)

Baeyer–Villiger oxidation of **1** was performed using *m*-chloroperbenzoic acid in chloroform. This was followed by hydroxide ion catalyzed hydrolysis of the product ester at pH 12 and then acidification of the reaction mixture. As seen from the data in Table I, the degradation of **1** gave rise to **2** with almost no loss of optical activity. The close correspondence between the specific rotation of the benzylsuccinic acid isolated from the degradation of (−)-**1** with the specific rotation for

Table I. The Optical Rotation of the 2-Benzylsuccinic Acids Derived from (+)-**1** and (−)-**1**

source	product	$[\alpha]_D$, deg
(+)-**1a**	(−)-**2**	−25.0 (*c* 3.87, ethyl acetate)[a]
(−)-**1a**	(+)-**2**	+23.9 (*c* 5.31, ethyl acetate)[a]
lit.[b]	L-(+)-**2**	+27

[a] Measured at 23 °C. [b] Reference 5. Measured at 20 °C.

(+)-**2** which possesses the *R* configuration establishes that (−)-**1**, the enantiomer exhibiting hydrogen-deuterium exchange at the activated methylene position, has the *R* configuration at the chiral center. That is, (−)-**1** corresponds essentially to a methylene analogue of *N*-benzoyl-L-phenylalanine.

Next, we turned to the determination of the absolute stereochemistry of the diasterotopic hydrogens (H_a and H_b) at the activated methylene group of (−)-**1**. Our approach to this aspect of our study was to examine the stereochemistry of the H_a and H_b protons relative to the hydrogen at the chiral methine position. If this relative stereochemistry could be determined, then, since the absolute stereochemistry at the chiral center of (−)-**1** is known, the absolute stereochemistry of the hydrogens at the *pro*-chiral 3-methylene position would also be established.

In the first stage of our investigation of the stereochemistry at the 3-methylene group, compound **1a** was converted to the corresponding *cis*- and *trans*-2,4-substituted γ-butyrolactones, **3a** and **4a**, for each of which the ^{1}H NMR signals for the protons at this methylene position can be easily distinguished. In particular, reduction of **1a** with sodium borohydride, followed by lactonization using dicyclohexylcarbodiimide (DCC), gave a mixture of the two isomeric γ-butyrolactones in a 4:1 ratio. (See eq 3.) The major component was found to be identical with the compound obtained by catalytic hydrogenation of 2-benzyl-4-*p*-methoxyphenylbut-2-en-4-olide (**5a**), prepared as illustrated in eq 4. Hussain et al.[6] have shown that in many cases the cis isomer of the 2,4-disubstituted γ-butyrolactone was obtained predominantly in the catalytic hydrogenation of 2,4-disubstituted but-2-en-4-olides. These workers[6] found though that in the hydrogenation of 2-methyl-4-phenylbut-2-en-4-olide roughly equal amounts of the *cis*- and *trans*-2-methyl-4-phenyl-γ-butyrolactones were obtained. However, they concluded that the cis:trans ratio seen in this instance probably does not reflect the stereoselectivity of hydrogenation at the 2,3 double bond in view of the competing hydrogenolysis of the C(4)-O single bond which was observed[6] (one of the products was 2-methyl-4-phenylbutyric acid). In our own experiments, where hydrogenation was performed over platinum oxide rather than over the palladium catalyst used by Hussain et al., only one saturated γ-butyrolactone was isolated, and, therefore, it was assigned the *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactone structure **3a**. On the basis of this identification, the major product resulting from the treatment of compound **1a** according to the sequence of eq 3 was also assigned the structure **3a** and thus the minor product has structure **4a**.

To assign the crucial ^{1}H NMR signals unequivocally, deuterium atoms were introduced at the 3-methylene position of 2-phenyl-4-*p*-methoxyphenyl-γ-butyrolactone. By the catalytic hydrogenation of **5b**, containing deuterium at the olefinic position, which was prepared from **1b** by the sequence of eq 4, *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactone-*3-d* (**3c**) was obtained. The deuterium atom in **3c** is located cis to the adjacent 2-benzyl- and 4-*p*-methoxyphenyl residues. The 3,3-dideuterio-γ-butyrolactones **3b** and **4b** were prepared from **1b** by the route illustrated by eq 3 above.

Figure 1 illustrates the relevant regions of the ^{1}H NMR

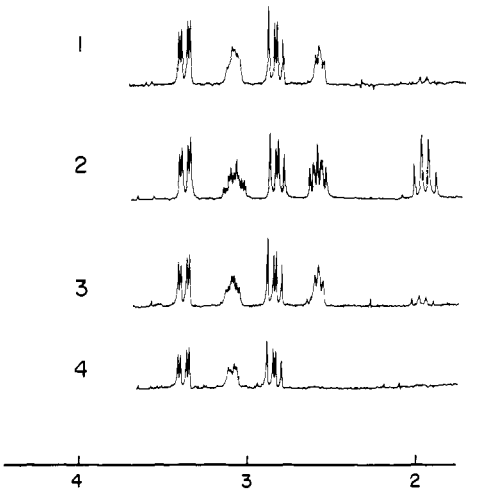
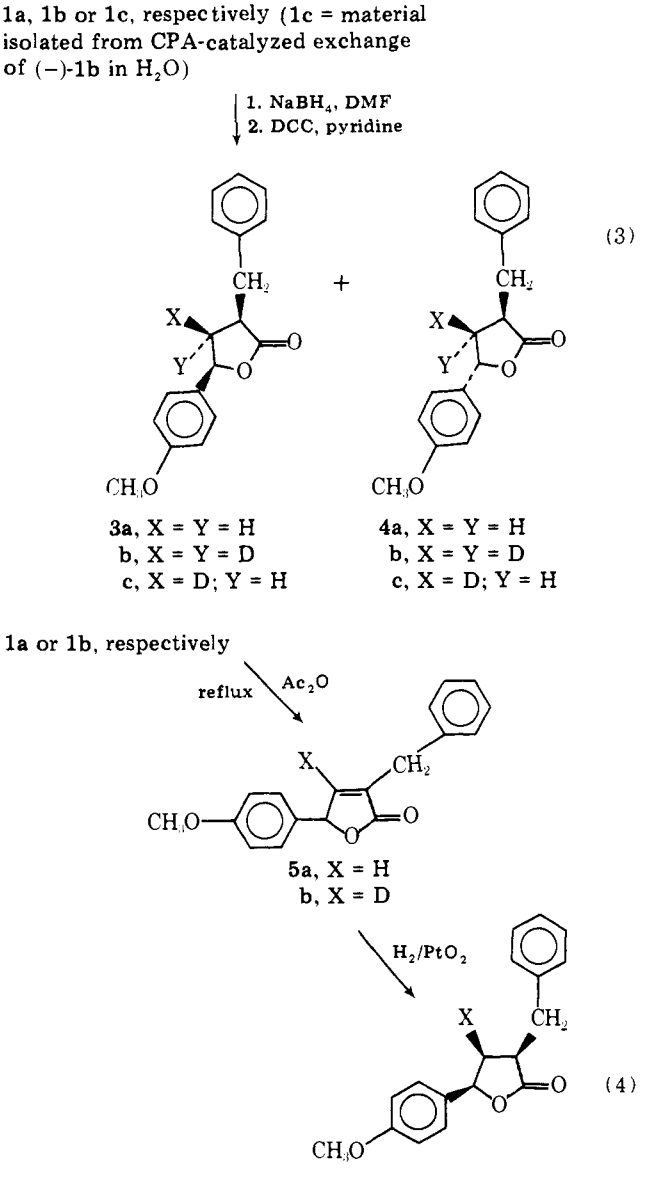


Figure 1. ^{1}H NMR spectra of *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones: (1) spectrum of lactone obtained from **1c**, the ketonic substrate isolated on incubation of a sample of (−)-**1b** with CPA_7 for 75 h at 25.0 °C; (2) spectrum of **3a**; (3) spectrum of **3c**; (4) spectrum of **3b**.



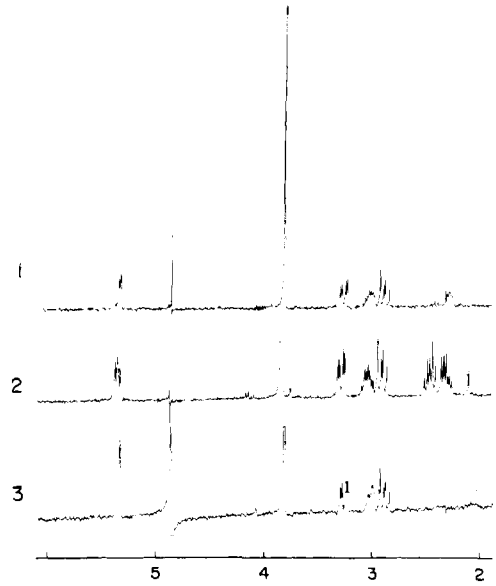


Figure 2. ^{1}H NMR spectra of *trans*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones: (1) spectrum of lactone obtained from **1c**; (2) spectrum of **4a**; (3) spectrum of **4b**.

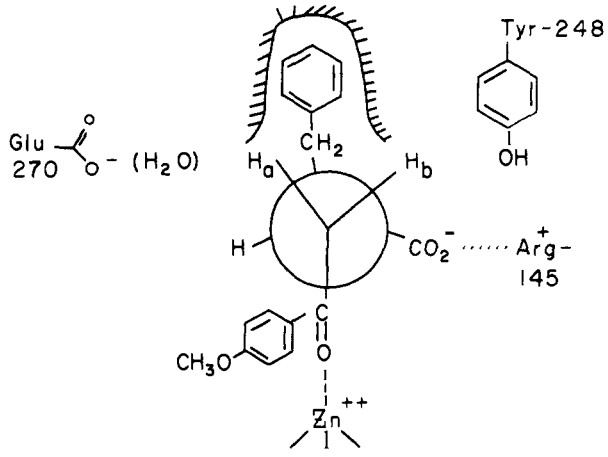


Figure 3. Postulated mode of binding of (−)-**1** to active site region of CPA leading to exchange at H_a position.

spectra of the various *cis*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones (**3a–c**) compared with that of the lactone obtained from **1c**, the ketonic substrate isolated on incubation of a sample of (−)-**1b** with CPA_γ for 75 h at 25.0 °C. The extent of exchange found for the **1c** isolated from this reaction was 74% for the H_a position and <5% for the H_b position. From the ^{1}H NMR spectra of **3a–c**, it can be readily seen that the 3-methylene proton trans to the 2-benzyl group gives rise to a peak at 2.55 ppm while the other methylene proton, cis to the 2-benzyl group, is responsible for the peak at 1.92 ppm. Furthermore, comparison of the ^{1}H NMR spectrum obtained for the lactone isolated from **1c** by the route of eq 3 with that of the lactone **3c** obtained from **1b** by the route of eq 4 clearly indicates that H_a, the proton introduced at 3-methylene position when (−)-**1b** is incubated with CPA, is trans to the 2-benzyl group in the former lactone.

Comparison of the ^{1}H NMR spectra of the *trans*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones **4a–c** led to the same conclusion. As is shown in Figure 2, the peaks due to the protons at the 3-methylene position of **4a** appear at 2.20 and 2.41 ppm. The signal observed at the higher chemical shift was assigned to the proton trans to the 2-benzyl group on the basis of the following facts. First, in several trans-2,4-disubstituted γ-butyrolactones, the proton at the 3-methylene position trans

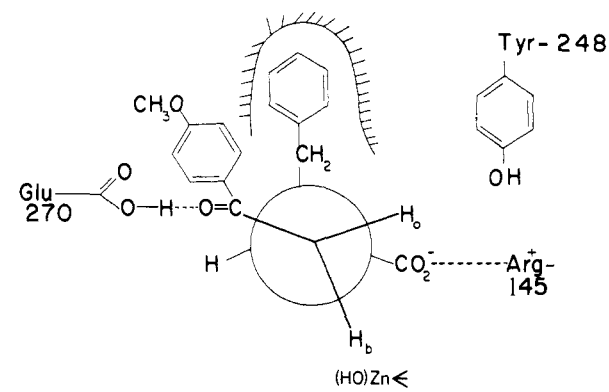


Figure 4. Alternative mode of binding of (−)-**1** to active site region of CPA. As discussed in the text of this manuscript, this binding scheme does not appear to account for the results obtained in the exchange reaction observed for (−)-**1**.

to the 2-alkyl or 2-aryl group appears at a slightly higher field than the proton cis to the 2 substituent.[6] Second, while the differences between the cis- and trans-vicinal coupling constants for the interaction between the 3-methylene protons and a 4-methine proton in most of the *trans*-2,4-disubstituted γ-butyrolactones were found to be small and sometimes negligible, to the extent that there are differences, the cis coupling constants are larger. Comparison of the signal at 5.33 ppm, which is assigned to the 4-methine proton on the carbon adjacent to the ring oxygen, observed in the spectrum of **4a**, with the corresponding signal in the spectrum obtained from the lactone **4c** allowed us to estimate the vicinal coupling constants for the interaction of the 3-methylene protons with the 4-methine proton: 5.5 Hz with the signal at 2.20 ppm, 7.7 Hz with the signal at 2.41 ppm. On the basis of this analysis, together with data in the literature on the ^{1}H NMR spectra of the *trans*-2,4-disubstituted γ-butyrolactones,[6] it is reasonable to assign the higher field signal to the 3-methylene proton positioned trans to the 4-methine proton, that is, trans to the 2-benzyl group. Thus, from the examination of the ^{1}H NMR spectra of the various *trans*-2-benzyl-4-*p*-methoxyphenyl-γ-butyrolactones, we have found that H_a, the proton introduced at the 3-methylene position when (−)-**1b** is incubated with CPA_γ, is trans to the 2-benzyl group in the trans-lactone system, exactly the same conclusion as we reached with the cis-lactone analysis.

In Figure 3 a schematic representation is given of the binding of (−)-**1** in the active site region of carboxypeptidase A assuming the same placement of this substrate as has been deduced for other substrates from X-ray crystallographic studies on peptide-enzyme complexes.[2] The stereochemical results that we have obtained indicate clearly that it is the *pro-R* hydrogen of the 3-methylene group which undergoes hydrogen–deuterium exchange at the active site of CPA_γ, and this is completely in accord with the picture drawn in Figure 3. On the basis of the stereochemical analysis, it is reasonable to conclude that the carboxylate group of Glu-270 is the functional moiety in the enzyme responsible for hydrogen abstraction and that the corresponding carboxyl group is the proton donor for the exchange reaction. Although evidence has been presented in the case of ester substrates that the carboxylate group of Glu-270 acts as a nucleophile leading to the formation of an anhydride species,[7] at this point, we cannot be certain whether or not the carboxylate group acts to remove the *pro-R* hydrogen of the 3-methylene group of (−)-**1** directly or through the intervention of a water molecule. Furthermore, while Figure 3 has been drawn with the carbonyl group of (−)-**1** bound directly to the metal ion, it is possible again that a water molecule might intervene. Experiments to explore this problem are currently under way in our laboratory.

In the case of peptide hydrolyses catalyzed by carboxypeptidase A it has been suggested that the phenolic hydroxyl of Tyr-248 may function to donate a proton to help generate the incipient leaving amino group.[2] Evidence has been obtained that for the hydrolysis of reactive esters catalyzed by CPA the corresponding donation of a proton from the Tyr-248 hydroxyl to aid formation of the leaving alcohol derived from the substrate is not important.[8] When the geometry of the ketonic substrate (−)-**1** bound as pictured in Figure 3 is considered, it can be seen that the tyrosine hydroxyl group cannot be functioning in its phenoxide form as the proton-abstracting group or in its phenolic form as the proton donor because this would predict exchange at the H_b (*pro-S*) position of the 3-methylene group.

In considering mechanisms of reaction at the active sites of hydrolytic enzymes, the problem is frequently faced whether the catalytic apparatus consists of functional groups in the arrangement A-H- -B or as A- -H-B, in other words, whether the acidic group is A-H and the basic group is B or the reverse holds. Similarly, if the trapping of an anhydride species in the CPA-catalyzed hydrolysis of the reactive ester *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate[9] were considered to be a special case, then there is a possibility (although it seems unlikely to us) that the binding of (−)-**1** to the active site region of CPA might be taking place according to the picture shown in Figure 4. If this were the situation, then the abstracting base would be zinc hydroxide and the carbonyl group of the substrate would be held in place by the carboxyl function of Glu-270. While several aspects of such a scheme are not attractive, it is hard to rule out on purely kinetic grounds. However, our stereochemical results clearly show that the CPA-catalyzed enolization reaction cannot be taking place with the substrate bound in this geometry. In particular, if zinc hydroxide were to remove the proton at the 3-methylene position of (−)-**1**, the hydrogen which would undergo the exchange reaction would be at the H_b (*pro-S*) position, contrary to our stereochemical observations. One last possibility is that (−)-**1** binds as shown in Figure 4 using the carboxyl of Glu-270 to hold the substrate's carbonyl group and that the phenoxide form of Tyr-248 acts as the abstracting base and the phenolic form of this residue as the proton donor. However, this scheme would lead to the prediction that k_{cat} would show a bell-shaped pH dependency, whereas in fact we have observed a sigmoidal dependency with no evidence that ionization of Tyr-248 plays any kinetically significant role.[10]

In summary, we have demonstrated that it is the *R* isomer of **1** which undergoes CPA-catalyzed hydrogen-deuterium exchange at its 3-methylene group and that it is the *pro-R* hydrogen which is susceptible to exchange at that position.

Acknowledgment. We are grateful to the National Institute of Arthritis, Metabolic and Digestive Diseases for support of this research. The NMR instrument used in this work was partly financed by the National Cancer Institute (PHS CA-14599) via the University of Chicago Cancer Research Center and by the National Science Foundation.

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Role of Active-Site Residues in the Catalytic Mechanism of Ribonuclease A

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Abstract: The electronic roles of His-12, Lys-41, Asn-44, His-119, the 119–120 backbone N–H, and Asp-121 in the transphosphorylation stage of the RNase A mechanism are assigned by combining known experimental facts with electronic structure theory calculations. His-12 is more likely to deprotonate $O_{2'}$, but in its absence Asn-44 may fulfill this role; His-119 activates the leaving group and facilitates in-line addition of $O_{2'}$; the 119–120 backbone N–H increases the electrophilicity of phosphorus; Gln-11···H_2O functions in a manner similar to the 119–120 backbone N–H; Lys-41 increases the phosphorus electrophilicity and stabilizes the trigonal-bipyramidal intermediate; Asp-121 may position the substrate, but does not act as a charge relay with His-119.

Although the catalytic mechanism of ribonuclease A (RNase A) has been studied extensively, uncertainty remains about various aspects. In this paper we attempt a description of the electronic rearrangements attendant to catalysis by combining the known experimental facts (including some unpublished X-ray data[1-3]) with results from electronic structure calculations. RNase A catalyzes the hydrolysis of a ribonucleic acid or a nucleotide ester by a two-stage mechanism.[4] The first stage, transphosphorylation,[5] involves addition of the 2′-OH group (on the 3′-ribose) to the phosphate group cleaving the ribonucleic acid chain at the 5′ end yielding a 2′–3′ cyclic phosphate and a free 5′-OH group (Figure 1). The second stage, hydrolysis,[5] involves addition of H_2O to the cyclic intermediate, yielding a terminal 3′-phosphate monoester (Figure 1). We are concerned primarily with stage one in the present article.

Viewed in its simplest terms, the transphosphorylation stage can be divided into several distinct steps.[4,6,7] In actuality it is likely that steps 2–5 are concerted. (1) Substrate is bound to the enzyme and the substrate–amino acid residue interactions prepare it for 2′-OH addition by making the phosphorus more electrophilic. (2) The 2′-OH group on the 3′-ribose is at least partially deprotonated, making it a better nucleophile. (3) During or after deprotonation, the phosphate group rotates

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