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MECHANISTIC STUDIES OF THE UROCANASE REACTION USING ^{1}H- AND ^{31}P-NMR SPECTROSCOPY AND THE SUBSTRATE ANALOGUE 2-METHYLUROCANATE

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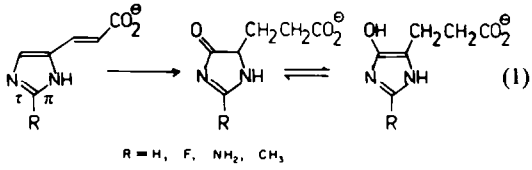
Abstract—The reaction of 2-methylurocanate with urocanase from *Pseudomonas putida* was monitored by ^{1}H-NMR spectroscopy at 500 MHz. The following conclusions were drawn: (i) 2-methylurocanate reacts 128 times more slowly with urocanase than does urocanate, (ii) no signals for the enol form of the produced 2-methylimidazolone propionate were detected, (iii) 2-methylimidazolone propionate is about 25 times more stable to hydrolysis than imidazolone propionate, (iv) the urocanase-catalysed exchange of the 5-proton of 2-methylurocanate with the solvent deuterium is 1.3 times faster than the overall reaction and (v) the non-enzymic exchange of the Me protons of 2-methylimidazolone propionate with solvent D takes place with a half life of 5.8 hr.

By ^{1}H-NMR spectroscopy it was shown that the urocanase reaction is reversible. At 8° and pD 6.3 1.6% of the total imidazolone propionate was converted into urocanate.

Apart from the pyrophosphate ester group of NAD$^+$ no phosphorylated groups could be detected in urocanase by ^{31}P-NMR spectroscopy.

INTRODUCTION

Urocanase catalyses the addition of water to urocanate in an unusual manner (eqn 1).



(1)

When considering a mechanism for this reaction, one has to account for the following facts: (i) two solvent protons are stereospecifically added to the side-chain double bond,[1,2] (ii) the O atom of the imidazolone ring originates from water[3,4] and (iii) urocanase contains a tightly bound NAD$^+$ that is essential for catalytic activity.[5,6]

The exact role of this NAD$^+$ is poorly understood. Lack of an H-transfer from position 5 of the substrate to any other position of the product[1,7–9] raised doubt concerning an H-carrier function of NAD$^+$ but did not exclude it strictly. Recent results of Matherly *et al.*[10,11] seem to support an initial nucleophilic attack of the τ N atom of the imidazole ring to position **4** of NAD$^+$.

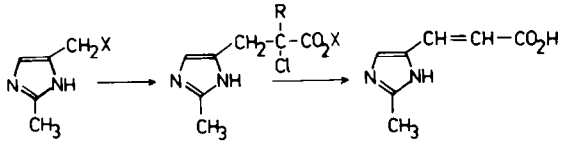
Kinetic ^{1}H-NMR spectroscopy of the urocanase reaction showed[7,8] that both the enol and the keto form of the product are formed. It has been additionally revealed[2,7–9] that urocanase catalyses the exchange of the 5-H atom of urocanate faster than the overall reaction.

Here we report on the reaction of urocanase with 2-methylurocanate as studied by kinetic ^{1}H-NMR spectroscopy at 500 MHz and on further details of the urocanase reaction.

RESULTS AND DISCUSSION

Synthesis of 2-methylurocanic acid. This was accomplished by following the synthetic scheme of Swaine.[12] In analogy to a classic synthesis[13] of 4-hydroxymethylimidazole fructose, *acetaldehyde* and ammonia were condensed to 2-methyl-4-hydroxymethylimidazole **1**. Unexpectedly, **1** was obtained concomitantly with 4-hydroxymethylimidazole (NMR!). This is explicable by partial degradation of the fructose to formaldehyde. After separation by ion exchange chromatography **1** was transformed via the chloride **2** into the chloromalonic ester **3** which gave in two conventional steps 2-methylurocanic acid (**5**). No attempts were made to optimize the yield; **5** was however for the first time fully characterized.

Kinetic ^{1}H-NMR-spectroscopy of the enzymic conversion of 2-methylurocanate. The kinetic measurements were carried out in a normal NMR-tube at a substrate concentration of about 15 mM in deuterated potassium phosphate buffer. After starting the reaction with urocanase, spectra were registered and stored in intervals of about 60 min. Three of these spectra are shown in Fig. 1. The first spectrum (A) taken after 50.8 min shows beside the signals of 2-methylurocanate a singlet at $\delta = 2.28$ arising from the 2-Me group of 2-methylimidazolone propionate and two complex signals at $\delta = 2.17$ and 1.89 whose relative intergrations are 3:1. Comparison of these signals with those of the keto form of imidazolone propionate[8] suggests that the signal at $\delta = 2.17$ arises



Scheme 1.

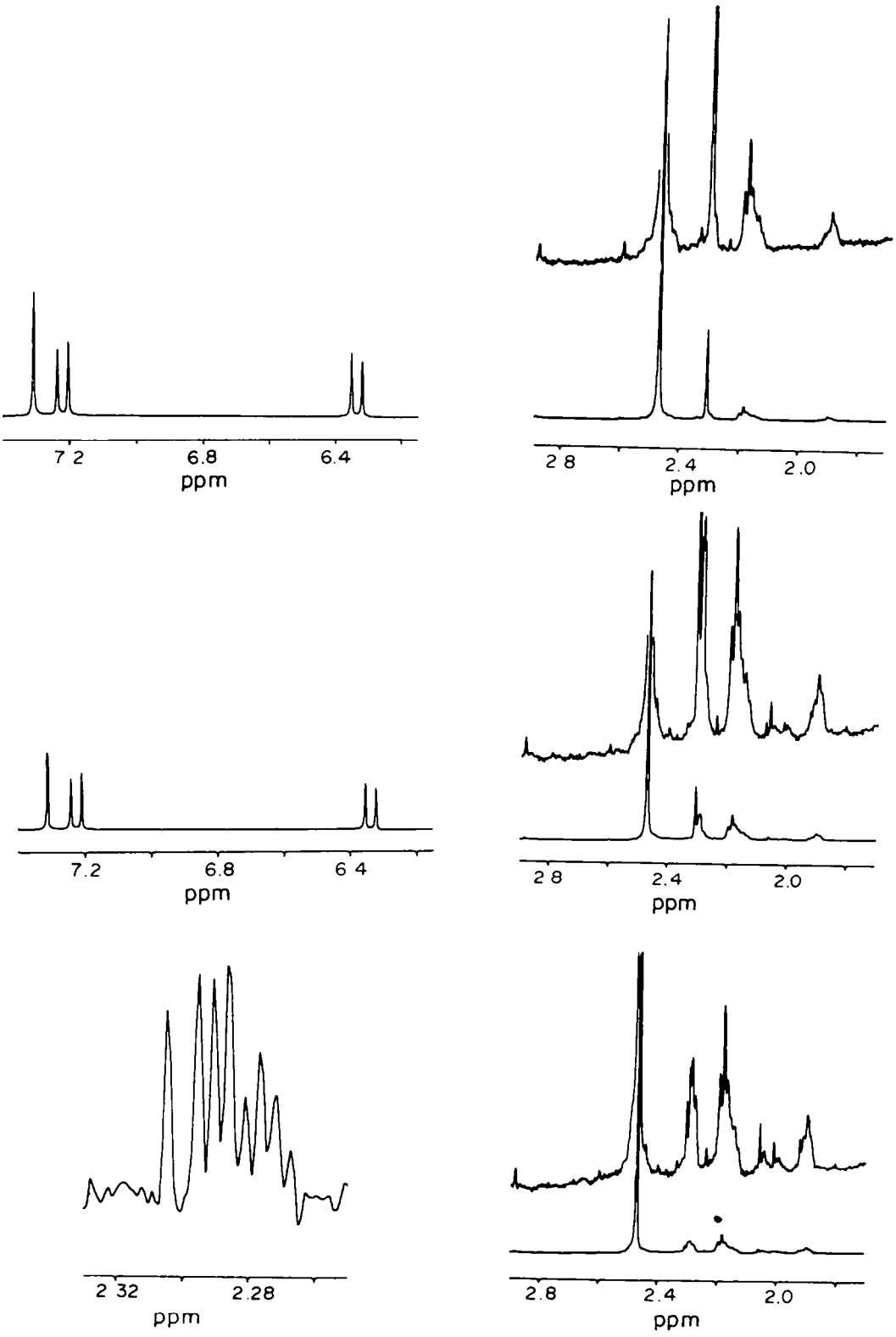


Fig. 1. The reaction of 2-methylurocanate with urocanase in deuterium oxide as monitored by ^{1}H-NMR spectroscopy at 500 MHz. The spectra were recorded 50.8 (A) 390 (B) and 1000 (C) min after the start of the reaction. In spectrum C the low-field portion is omitted and an expansion of the methyl region at enhanced resolution is shown on the left side. For further details and discussions see text.

from the two diastereotopic α-protons of (4*R*)- and (4*S*)-2-methylimidazolone propionate plus from the β-proton of one of these species (Fig. 1). The β-proton of the other stereoisomer resonates at $\delta = 1.89$. Integration of all signals reveals that about 21% of the substrate has been converted into 2-methylimidazolone propionate after 50.8 min.

In contrast to imidazolone propionate 2-methylimidazolone propionate does not appear to be in a well-balanced equilibrium with its enol form, at least no corresponding signals ($\delta = 2.39$ and 2.69)[8] are detectable. Since the ratio of (4*R*)- and (4*S*)-2-methylimidazolone propionates is 1:1, urocanase produces either a racemate, or more likely racemization occurs via the enol form. The data are not sufficient to decide whether the enol or the keto form is the primary product. This problem will be discussed later.

Spectrum B of Fig. 1 was recorded after 390 min when the enzymic reaction had come to an end. Integration shows that at this time about 42.5% conversion was achieved. Note that next to the methyl singlet at $\delta = 2.28$ a triplet has appeared. The upfield chemical shift corresponds to a typical deuterium isotope effect suggesting an exchange of the Me protons with solvent D. Since this exchange continues after the end of the enzymic reaction (*vide infra*) it cannot be enzyme-catalysed. The probable mechanism of this non-enzymic exchange is illustrated in Scheme 2. Via protonation of the carbonyl oxygen and proton abstraction from the 2-Me group **6a** is tautomerized into **6b** which in turn incorporates one D during the reverse reaction. Compound **6c** may be the subject of a further exchange as becomes evident from spectrum C of Fig. 1. The signal system in the Me region appears at enhanced resolution as a singlet ($\delta = 2.305$), a triplet ($\delta = 2.290$) and a quintet ($\delta = 2.275$) arising from the undeuterated monodeuterated and dideuterated species, respectively. The rate constant for the exchange of all three H atoms is $k = 3.35 \times 10^{-5}\,s^{-1}$ corresponding to a half life time $t/2 = 5.8$ hr.

Integration of the low-field portion of spectrum B reveals that the singlet at $\delta = 7.31$ decreases faster than the doublet at $\delta = 7.23$. This is due to an enzyme-catalysed exchange of the 5-proton with solvent deuterium as observed also with the natural substrate urocanate.[2,7-9] Whereas in the case of urocanate the signal for 5-H decreases 1.6 times faster than the signals for β-H, this factor is 1.3 in the case of 2-methylurocanate at 27° and pD 7.75.

Although 2-methylimidazolone propionate is more stable against hydrolysis than imidazolone propionate, it is evident from spectra B and C of Fig. 1 that it slowly deteriorates under the experimental conditions.

The two diastereotopic 3-protons of the two enantiomeric isoglutamine derivatives[8] can be observed due to their signals at $\delta = 1.90$ and 2.04. The corresponding α-proton and Me signals are hidden under the signal at $\delta = 2.17$. After 1000 min (spectrum C, Fig. 1) about 27% of the product was hydrolysed to N-acetylisoglutamine corresponding to $k = 5.25 \times 10^{-6}\,s^{-1}$ and a $t/2$-value of 37 hr. This compares with 75 min, the half life of imidazolone propionate under similar conditions.

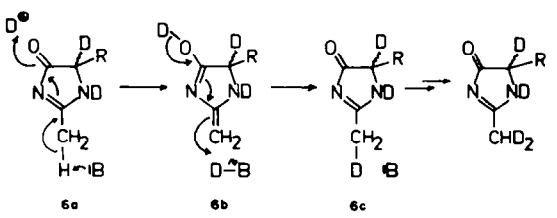
Substrate specificity of urocanase. Apart from urocanate hitherto only 2-fluoro- and 2-amino-urocanate were reported to be reluctant substrates of urocanase from *Pseudomonas putida*, reacting 150 and 1000 times, respectively, more slowly than the natural substrate.

Many other analogues are non-substrates or inhibitors (e.g. imidazole propionate). 2-Methylurocanate was reported[14] to be inert with urocanase from cat liver but the assay method might have been not sensitive enough. (Monitoring the reaction by UV spectroscopy prohibits the use of high substrate concentrations). In our ^{1}H-NMR measurements (Fig. 1) 5.5 U urocanase converted 2.28 μmol 2-methylurocanate during 119 min. Accounting for the isotope effect in D_2O ($k_H/k_D = 2.25$ for urocanate as substrate) one can calculate that 2-methylurocanate reacts about 128 times more slowly than the natural substrate under the conditions of the ^{1}H-NMR experiment (Fig. 1).

The reversibility of the urocanase reaction as investigated by ^{1}H-NMR spectroscopy. Imidazolone propionic acid was prepared enzymically by a modified[8] method of Hassal and Greenberg.[4] After incubation with urocanase in buffered D_2O the ^{1}H-NMR spectrum of the equilibrium mixture was recorded at 250 MHz (Fig. 2). Integration of the singlets at $\delta = 7.55$ and 8.06 as well as the doublets at $\delta = 6.29$ and 7.10 gave the relative amounts of the enol and keto forms of imidazolone propionate as well as that of urocanate. Furthermore, the amount of the hydrolysis product, N-formylisoglutamine, could be determined by integration of the singlet at $\delta = 7.98$. By the choice of the temperature (8°) and the pD (6.3) the irreversible hydrolysis of imidazolone propionate was reduced as far as possible, although it was not deleterious for the equilibrium measurements. Under these equilibrium conditions the ratio of the keto and enol forms of imidazolone propionate was 1.18. The amount of urocanate produced in the reverse reaction was 1.6% of the total amount of imidazolone propionate (keto plus enol form). The equilibrium constant (K′) (see eqn 2) of the urocanase reaction can be calculated as 33.8 or as 28.7 depending on whether the keto or the enol form is the genuine product. The temperature and pH dependence of the equilibrium constants both of the keto-enol tautomerization and of the urocanase reaction could give clues to the nature of the genuine product. Investigations of this kind are under way

$$K[H_2O] = K' = \frac{[\text{imidazolone propionate}]}{[\text{urocanate}]} \qquad (2)$$

The urocanase equilibrium was first measured using [^{14}C]-imidazolone propionate.[15] About 5% of the radioactivity was found in urocanate. More exact values were obtained by Cohn *et al.*[16] using UV



Scheme 2.

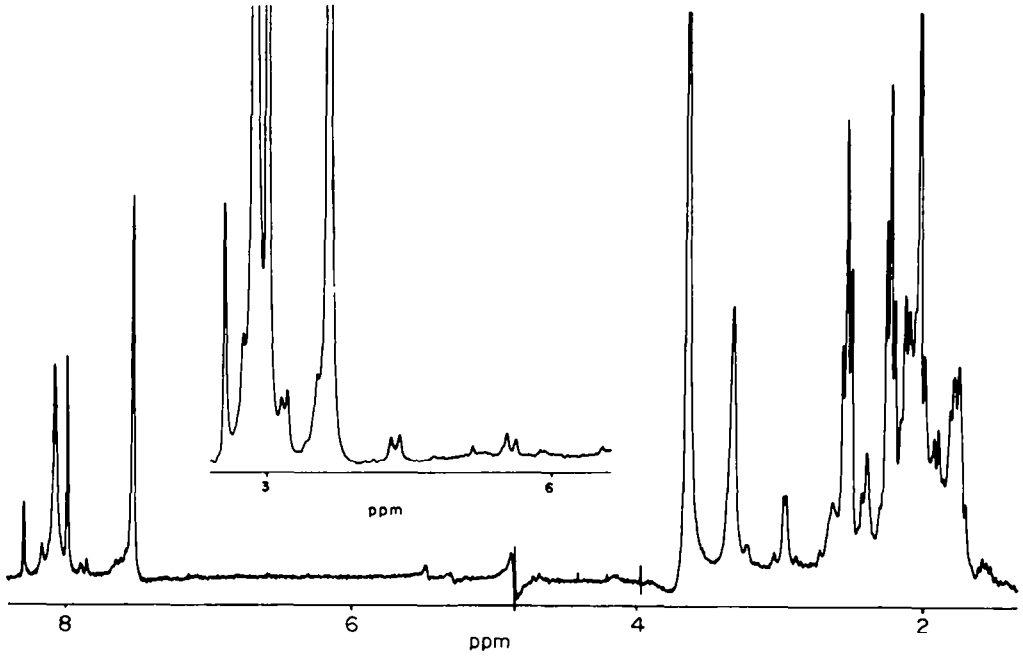


Fig. 2. ^{1}H-NMR spectrum of imidazolone propionate in deuterium oxide at 250 MHz. The partial spectrum above was recorded after equilibration with urocanase. The reaction mixture consisted of 0.25 ml 0.2 M deuterated potassium phosphate buffer (pD 7.5), 0.18 ml 3 M NaOD, 92 μmol imidazolone propionic acid in 0.15 ml deuterium oxide/DCl and 2.5 U urocanase in 70 μl deuterated potassium phosphate buffer. The final pD was 6.3. The spectra required 150 transients, the HOD signal was taken as a reference ($\delta = 4.86$) and was suppressed by gated decoupling. For discussion see text.

spectroscopy. They found that 1.38% of the total imidazolone propionate was converted into urocanate at pH 7.5 and 25°. This compares favourably with our value measured under somewhat different conditions (*vide supra*).

^{31}P-NMR *Spectrum of urocanase.* A number of enzymes are regulated by reversible phosphorylation by cyclic AMP-dependent protein kinases.[17] According to recent reports[18,19] urocanase from rat liver belongs also to this group of enzymes. Therefore it was of interest whether a phosphorylated group could be detected in urocanase from *P. putida.*

Apart from labelling with radioactive ^{32}P ^{31}P-NMR spectroscopy is the method of choice for tracing phosphate groups in a protein. The ^{31}P-NMR spectrum of a urocanase preparation from *P. putida* (Fig. 3) shows a large signal at $\delta = -8.76$ (external H_3PO_4, $\delta = 0.0$) which can be assigned to the NAD$^+$ tightly bound to urocanase.[5,6] A smaller peak at $\delta = -10.37$ might arise from either NADH or from the diphosphate group of a modified NAD$^+$ (e.g. a nucleophilic addition product thereof). Finally the small signal at $\delta = +2.84$ might come from traces of inorganic phosphate.[20]

In a literature reference[21] one finds for the phosphate group of phosphoserine and phosphothreonine signals at $\delta = +4$ and $+2.4$, respectively.

Our results suggest that urocanase from *P. putida* has no phosphorylated functional group and is therefore not regulated by a protein kinase system.

EXPERIMENTAL

^{1}H-NMR and ^{31}P-NMR spectra were recorded on a Bruker WH 90, WP 200, WH 250, WH300 or WM 500 spectrometer. ^{1}H δ-values are given in ppm relative to trimethylsilyl-(2-$^2H_2$, 3-$^2H_2$)propionate ($\delta = 0.0$), coupling constants are in Hz. UV spectra were taken on a Cary 14 spectrophotometer. Mps are uncorrected. Urocanase (specific activity 1.3 U/mg) was isolated from *Pseudomonas putida* (ATCC 11299), according to described methods.[6,22]

Synthesis of 2-methylurocanic acid[12]

Compound **1a** (m.p. 169°) and hydrochloride **1b** were obtained according to the method of Totter and Darby.[13] **1b** (500 mg) was chromatographed on a Dowex column (50 W × 4, 200–400 mesh, H^+ form, 30 × 2.5 cm). After washing the column with 0.033 M HCl (14 l) **1b** was eluted with 0.04 M HCl (5 l). Fractions containing **1b** but less than 5% contaminating 4-hydroxymethylimidazole (^{1}H-NMR!) were combined, yield: 154 mg. ^{1}H-NMR (D_2O, 250 MHz): $\delta = 2.68$ (*s*, 3 H), 4.72 (*s*, 2 H), 7.31 (*s*, 1 H).

2-Methyl-4-chloromethyl imidazole hydrochloride **2** was prepared from 154 mg (1.04 mmol) **1b** and 185 mg (1.55 mmol) thionylchloride in 3 ml dry benzene (75°, 2 hr). The solid product was separated from the solvent and dissolved in 4 ml dry EtOH. This soln was used in the next step.

α - Chloro - α - ethoxycarbonyl - β - (2 - methylimidazolyl-propionic acid **3**. A soln of 50 mg (2.08 mmol) NaH in 5 ml dry EtOH was stirred at 0° and treated dropwise with a soln of 201.8 mg (1.04 mmol) chloromalonic acid diethylester (EGA-Chemie, D-7924 Steinheim, 98%) in 4 ml dry EtOH. Further stirring for 15 min was followed by the addition of the above ethanolic soln of **2**. After 0.5 hr the precipitated NaCl was removed by filtration through celite. Evaporation of the solvent yielded a yellowish film which was used in the next step without further characterization.

α - Chloro - β - (2 - methylimidazolyl)propionic acid **4** was obtained by heating the above product (**3**) with 5 ml conc HCl for 6 hr to 110°. Removal of the solvent resulted in a yellowish crystalline solid. ^{1}H-NMR (D_2O, 90 MHz):

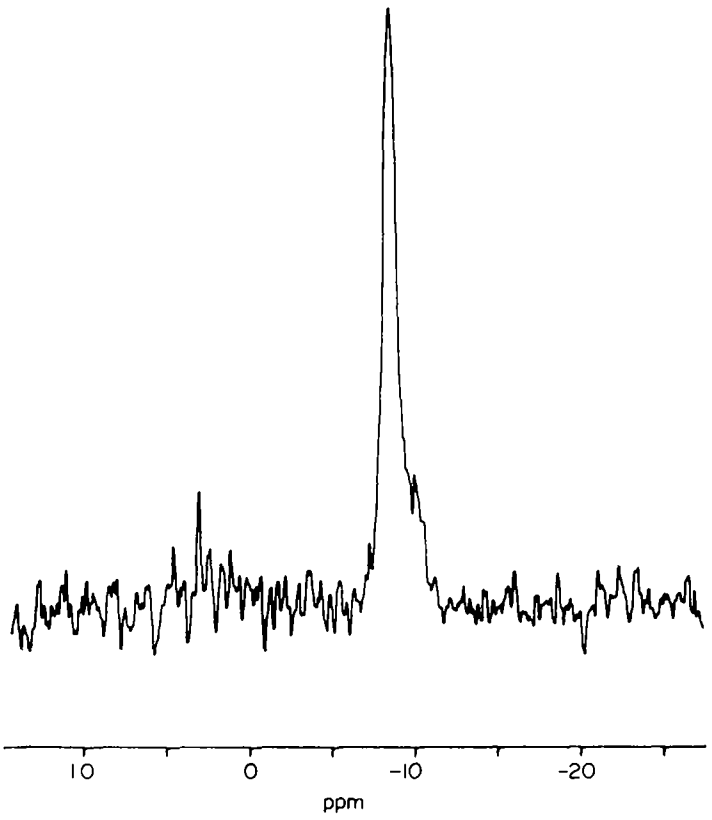


Fig. 3. ^{31}-*P-Spectrum of urocanase at* 81.02 MHz. 135 U (0.56 μmol) urocanase in 1.5 ml Tris/HCl buffer (pH 7.3) was used and 10% deuterium oxide was added for lock. 85% H_3PO_4 was the external reference ($\delta = 0.0$). The spectrum was ^{1}H-decoupled and required 37,000 transients with a recycle time of 1.4 s. The temperature was kept at 25°C. For discussion see text.

$\delta = 2.63$ (*s*, 3 H), 3.44 (*d*, $J = 6$ Hz, 2 H), 4.86 (*t*, $J = 6$ Hz, 1 H), 7.26 (*s*, 1 H).

2-Methylurocanic acid **5**. The crude product **4** was dissolved in 5 ml 40% soln of Et_3N in water (w/w). The soln was heated to 60° for 48 hr in a sealed ampule, then transferred to an open vessel and refluxed for 6 hr. Removal of the solvent resulted in a residue that was submitted to chromatography on Dowex-50 (*vide supra*). Fractions containing **5** were adjusted to pH 4.7 with 0.1 M NaOH. **5** crystallized upon cooling to 0°, yield: 7 mg (41.2 μmol, 4% overall), m.p. 207–208 (Ref. 12: 206°) UV (0.2 M potassium phosphate buffer, pH ≙ 7.5): $\lambda_{max}(\epsilon) = 281$ nm (16200 M^{-1} cm^{-1}). ^{1}H-NMR (D_2O, 300 MHz, pD 7.9): $\delta = 2.44$ (*s*, 3 H), 6.31 (*d*, $J = 16$ Hz, 1 H), 7.22 (*d*, $J = 16$ Hz, 1 H), 7.17 (*s*, 1 H). (Found: C 49.1, H 5.39, N 15.81. Calc for $C_7H_8O_2N_2 \times H_2O$ (m.w. 170): C 49.4, H 5.88, N 16.47%.)

Monitoring the reaction by ^{1}H-NMR *spectroscopy at* 500 MHz. The following soln was prepared in an NMR-tube: 1 mg (5.9 μmol) 2-methylurocanic acid × H_2O, 0.37 mg K_2CO_3, were dissolved in 0.25 ml deuterated potassium phosphate buffer (0.2 M). 0.01 ml Trimethylsilyl-(2-$^2H_2$, 3 $^2H_2$) propionate solution in ^2H_2O and 0.15 ml urocanase soln (containing about 5.5 U) were added. The NMR-tube was filled with argon. The pD of the mixture was 7.75 (pH meter reading 7.35) and the temp was kept at 27°. After certain time intervals spectra were recorded. The last spectrum was taken after 17 hr. The enzymic reaction came to an end after 186 min. Each spectrum resulted from the accumulation of 64 transients for a total time of 7.77 min.

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