Tetrahedron Letters,Vol.23,No.41,pp 4221-4224,1982 0040-4039/82/414221-04$03.00/0
Printed in Great Britain

SOME REACTIONS OF PHENOLIC MALEAMIC ACIDS RELEVANT TO CARBOXYPEPTIDASE CATALYSIS

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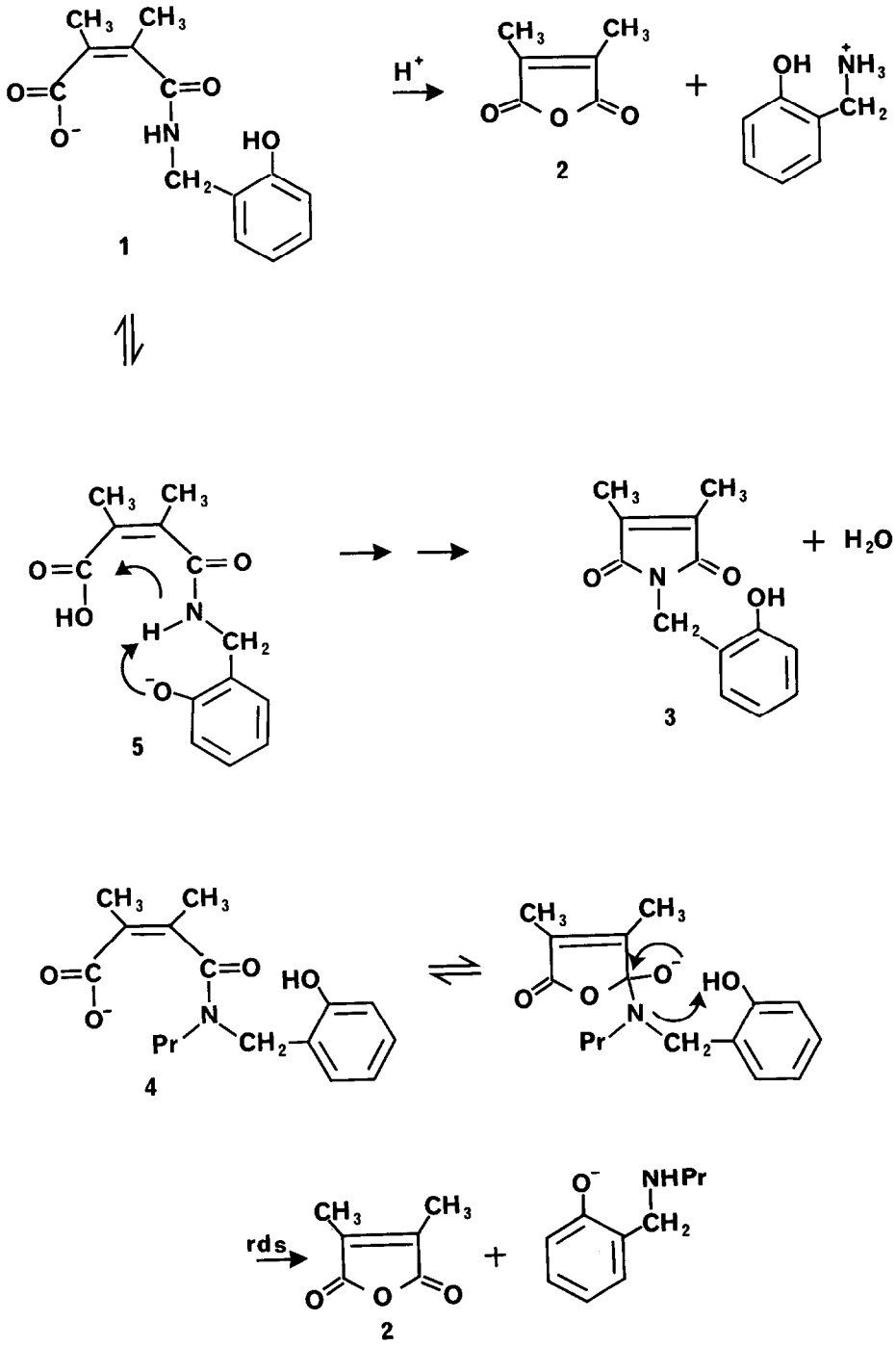
Summary: In neutral aqueous acetonitrile, N-o-hydroxybenzyl-2,3-dimethylmaleamic acid is rapidly converted to N-o-hydroxybenzyl-2,3-dimethylmaleimide, while N-propyl-N-o-hydroxybenzyl-2,3-dimethylmaleamic acid is converted to dimethylmaleic anhydride; both reactions are catalyzed by the phenolic groups.

The enzyme carboxypeptidase A cleaves peptides with the assistance of three catalytic groups: the carboxylate ion of a glutamate residue, the phenolic hydroxyl of a tyrosine, and a zinc ion. For this reason we have been investigating molecules in which a carboxylate ion and a phenol could cooperate in cleaving an intramolecular amide linkage. A few years ago a report from our laboratory[1] described the change which occurred when one such species, compound (1), was allowed to react in 1 M H_2O/CH_3CN under acidic and neutral conditions. Under acidic conditions the well-precedented[2] conversion of maleamic acid (1) to the maleic anhydride (2) and amine was observed, but at neutrality a new process took over in which the anhydride was not produced. This process was promoted by the phenolic group, and was proposed[1] to be a hydrolysis of the amide group by a general base mechanism catalyzed by carboxylate and phenol. On further investigation, we find that the products of this new process were mis-identified, and the reaction at neutrality is actually a closure of the maleamic acid (1) to the maleimide (3). This closure[3] can be blocked by N-propylation in compound (4); under both neutral and acidic conditions (4) reacts to form the anhydride (2). At neutrality, the maleimide formation from (1) and the anhydride formation from (4) are catalyzed by the phenol groups.

Maleamic acids (1) and (4) were obtained, as the amine salts, by reaction of two equiv. of the appropriate amine[4] with dimethylmaleic anhydride. An authentic sample of the maleimide (3), (m.p. 79-80°, M + 1 = 232, ^{1}H NMR in $CDCl_3$ δ 1.98 (6H, s), 4.63 (2H, s), 7.50 to 6.73 (4H, m); UV λ_{max} in H_2O, 270 nm (ε 8,000) 310 nm (ε 500); IR 1700 cm^{-1}) was obtained from (1) by heating[3(c)] (150°, 3 hrs). Kinetic studies at 25.0° were performed, as described[1], in 1 M


CH3 CH3
O=C C=O
O- HN HO
CH2
1
H+
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OH NH3+
CH2
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+ H2O
3
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NHPr

H_2O/CH_3CN with 5×10^{-3} M HOAc and 5×10^{-4} M KOAc (10:1, acidic), 5×10^{-4} M HOAc and 5×10^{-3} M KOAc (1:10, neutral), and 5×10^{-3} M KOAc with no added HOAc (referred to as 1:50, neutral). The absorbance was followed at 250 nm, which is λ_{max} for dimethylmaleic anhydride. In all cases the kinetic runs gave good first-order plots for at least two half lives.

In the acidic buffer, compound (4) underwent ring closure to form the anhydride (2) (increase in absorbance) with $k = 12.0 \pm 0.5 \times 10^{-4}$ s^{-1}; this decreased by only a factor of 5, to $k = 2.5 \pm 0.5 \times 10^{-4}$ s^{-1}, in the neutral (1:10 and 1:50) buffers, in which closure to form the anhydride was followed by a slower decrease in absorbance as the anhydride hydrolyzed. Since we had found[1] that in maleamic acids without a phenolic catalytic group the rates of ring closure decrease by ca. 10^3 in proceeding from the 10:1 to the 1:50 buffer system, the phenolic group in (4) is contributing more than a factor of 10^2 to the ring-closure process at neutrality. This catalysis is presumably due to protonation of the leaving group nitrogen by the phenol, as shown.

In the acidic buffer, compound (1) also underwent closure to dimethylmaleic anhydride (2) with an increase in absorbance at 250 nm, as we had reported.[1] We find that this process has $k = 20.0 \pm 0.5 \times 10^{-4}$ s^{-1} (rept'd.[1] 45.5×10^{-4} s^{-1}). The product (2) was further identified by high performance liquid chromatography (HPLC). When the reaction mixture was allowed to stand, this first anhydride product (2) disappeared and was replaced by the maleimide (3), identified by HPLC and UV (broad maximum at 310 nm). In the neutral buffers the anhydride (2) was not formed, even as a transient intermediate. (We have reported[1] that (2) could not be trapped with amines present in the reaction mixture which successfully trap authentic (2) under the same conditions, and this has been confirmed.[5]) Instead, it underwent a new process with $k = 1.7 \times 10^{-4}$ s^{-1}, in agreement with our previous report[1] of $k = 1.9 \pm 0.1 \times 10^{-4}$ s^{-1}, with a steady decrease in absorbance at 250 nm. The product of this process was the maleimide (3), identified by HPLC and UV spectra. It was formed quantitatively (HPLC) at the expected rate determined from the UV kinetic studies.

As reported[1], this new reaction of (1) at neutrality is ca. 70 times as rapid as is the disappearance of an analog of (1) without the phenolic group under the same conditions. Thus at neutrality the phenol group catalyzes maleimide formation. The most likely mechanism is that shown, involving the kinetically equivalent carboxylic acid/phenoxide state (5) of the substrate. Less likely alternatives would involve reversible formation of a tetrahedral intermediate, with phenol catalysis in a subsequent rate-determining step. Curiously, in acid the closure of (1) to the anhydride is <u>faster</u> than is the closure of (4), but at neutrality (4) continues to form anhydride while (1) substitutes a new process, closure to the maleimide; however, the reaction of (1) is now <u>slower</u> than the reaction of (4), so the anhydride formation from (1),

no longer the major process, must have a greatly depressed rate compared with that of (4).

We have proposed[6] that the cleavage of peptide substrates by carboxypeptidase A occurs by a general base mechanism, while Makinen[7] has demonstrated that cleavage of an ester substrate occurs by a nucleophilic mechanism, with formation of an intermediate anhydride. It is now clear that the chemistry of (1) is not related to the general base mechanism for catalysis by carboxypeptidase A, as had been suggested[1]. Furthermore, compound (4) continues to use the nucleophilic mechanism, with phenol catalysis, even at neutrality. Thus other models must be sought for the mechanism we have proposed[6] for the cleavage of amides by a carboxylate and a phenol group (and a zinc atom) in carboxypeptidase catalysis.[8]

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8. We wish to thank Drs. Wayne Delker and Dao-Dao Zhang for preliminary work to this study. Financial support of this work by the National Institutes of Health and the award of a postdoctoral fellowship to Jik Chin from the Natural Sciences and Engineering Research Council of Canada are gratefully acknowledged.

(Received in USA 24 June 1982)