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Aspartic Proteinases: Fourier Transform Infrared Spectroscopic Studies of a Model of the Active Side

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ABSTRACT We synthesized and studied by Fourier transform infrared spectroscopy nine monosalts of diamides as models for the active side of aspartic proteinases. One compound, the monosalt of *meta*-aminobenzoic acid diamide of fumaric acid (*m*-FUM), shows the same biological activity as pepsin with regard to the splitting of peptide bonds of the Pro-Thr-Glu-Phe-Phe(4-NO_2)-Arg-Leu heptapeptide. The monosalt of *m*-FUM forms with oxindole a complex in which the carboxylic acid group of the monosalt of *m*-FUM is strongly hydrogen bonded with the O atom of the peptide bond of oxindole. When one water molecule is added to this complex, the strong field of the carboxylate group destabilizes an O-H bond of the water molecule. The distorted water molecule attacks the carbon atom of the peptide group, and the water proton transfers to the peptide N atom. Simultaneously, the C-N bond of the amide group is broken. Hence it is demonstrated that the catalytic mechanism of aspartic acid proteinases is a base catalysis. The results show that for this catalytic mechanism there are sufficient carboxylic and carboxylate groups, as well as a water molecule in the correct arrangement. It was also demonstrated with other monosalts of dicarboxylic acids that well-defined steric conditions of the carboxylic acid and the carboxylate group must be fulfilled to show hydrolytic activity with regard to oxindole molecules.

INTRODUCTION

Aspartic proteinases form a widely distributed class of proteolytic enzymes. They are found in vertebrates, fungi, plants, and recently in retroviruses (Davies, 1990). They are all characterized by pH optima in the acidic range and inhibition by pepstatin, a naturally occurring inhibitor from streptomyces. Of essential importance for the catalytic mechanism of all aspartic proteinases is the presence of a well-conserved aspartic acid as well as aspartate residues in the active center.

The current knowledge of the aspartic proteinases is based on detailed x-ray structural studies of these enzymes. The x-ray structures of the mammalian enzymes pepsin (Cooper et al., 1990; Sielecki et al., 1990), chymosin (Newman et al., 1991), and human renin (Sielecki et al., 1989); the fungal enzymes endothiapepsin (Blundell et al., 1990), penicillopepsin (James and Sielecki, 1985), and rhizopuspepsin (Suguna et al., 1987) and their complexes with inhibitors (James and Sielecki, 1985; James et al., 1992; Suguna et al., 1987; Veerapandian et al., 1992) have shown that in the active site one aspartic acid residue, one water molecule, and one aspartate residue are present in close vicinity. The location of protons, however, cannot be determined directly by x-ray diffraction.

In some cases a strong homoconjugated hydrogen bond $(O{\cdot\cdot}H{\cdot\cdot}O)^-$ between Asp 215 and Asp 32 (pepsin numbering) has been postulated (Pearl and Blundell, 1984; James and Sielecki, 1985). An alternative arrangement was suggested by Suguna et al. (1987). They assume that the proton is located at one Asp residue and the nucleophil is a central water molecule. Recent studies of James et al. (1992) and Veerapandian et al. (1992) support this assumption. As a reaction mechanism a base catalysis is proposed (Suguna et al., 1987; James et al., 1992; Veerapandian et al., 1992). But an acid catalysis due to the presence of a (H_3O^+) hydronium ion was not unambiguously excluded (James and Sielecki, 1985). Recently it was shown by a Fourier transform infrared (FTIR) spectroscopic study that no homoconjugated hydrogen bond exists between the catalytic aspartic acid and the aspartate groups (Iliadis et al., 1994). Furthermore, this study has suggested that with pepsin, the catalytic mechanism is probably a base catalysis by a water molecule present in the active side (Iliadis et al., 1994).

Of particular interest for those proton shifts during catalysis is the fact that a carboxylic acid-water carboxylate system is a hydrogen-bonded pathway with large proton polarizability (Eckert and Zundel, 1980). Thus the proton can easily be shifted by changes of local fields in this hydrogen-bonded system.

To obtain detailed information on the stereochemistry and the intermolecular forces in the active side of the aspartic proteinases, a series of model molecules resembling the active side of pepsin were synthesized. In these models the carboxylic and the carboxylate groups are kept in close vicinity while the distance between them is varied. For this purpose *ortho*-, *meta*-, and *para*-aminobenzoic acids were used for the carboxylic residues to simulate the catalytic Asp residues. The distance between the carboxylic groups of the spacer was varied in the following sequence: isophthalic, fumaric, 2,3-dimethylsuccinic, and terephthalic acid.

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We studied the catalytic activity of all of these model molecules with regard to peptide bond splitting and studied by FTIR spectroscopy the nature of the splitting mechanism.

MATERIALS AND METHODS

Porcine pepsin and substrate

Pepsin was purchased from Boehringer-Mannheim GmbH, and the chromophoric substrate Pro-Thr-Glu-Phe-Phe(4-NO_2)-Arg-Leu from Bachem Biochemical GmbH (Heidelberg, Germany).

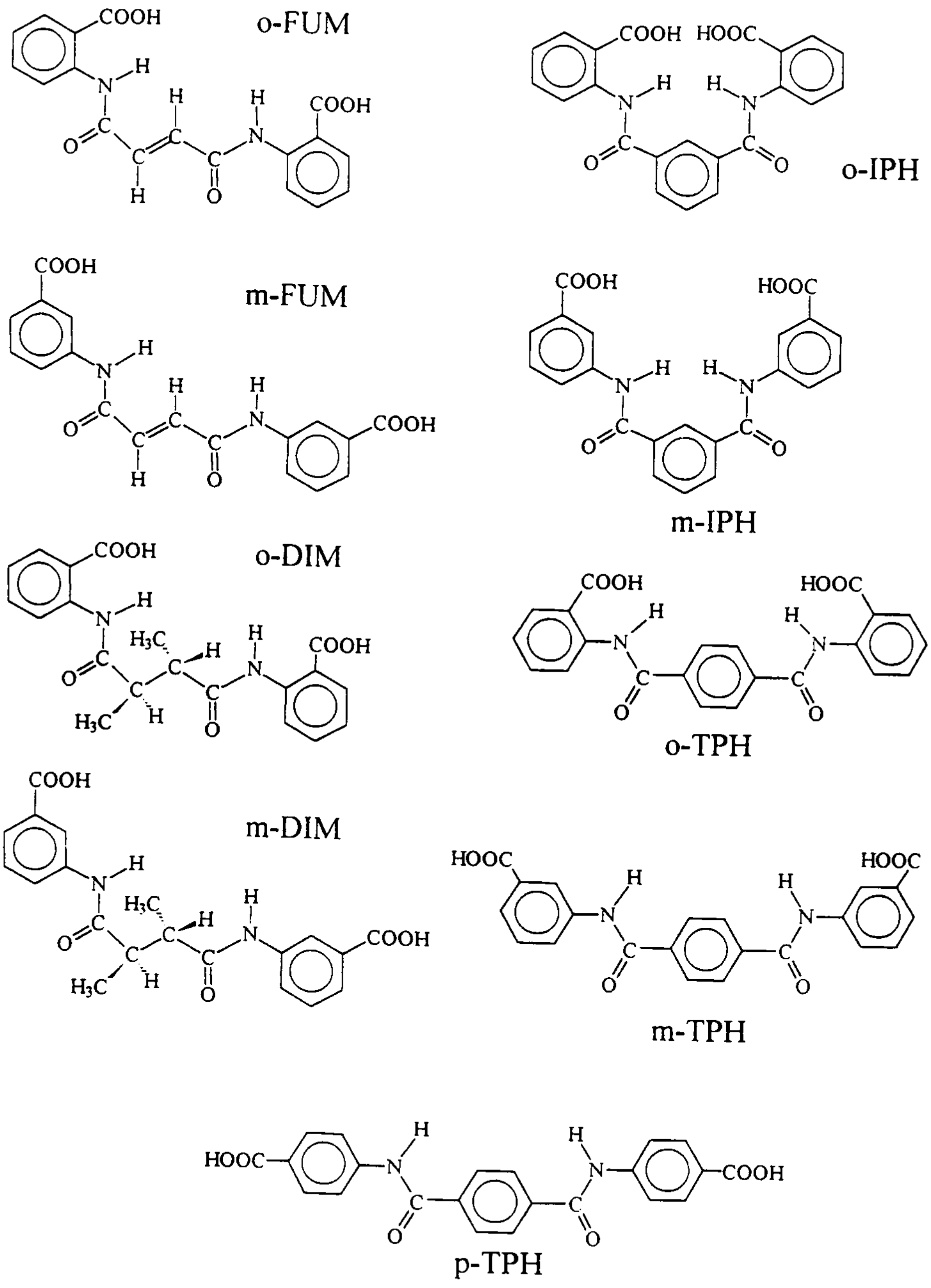
Materials

All chemicals for the synthesis of model compounds 1–9 (Scheme 1), *meta*-aminobenzoic acid, *para*-aminobenzoic acid, fumaryl dichloride, oxalyl chloride, isophthalyl dichloride, and terephthalyl dichloride were purchased from Sigma-Aldrich Chemie (Munich, Germany) and were used without further purification.

Synthesis of the model compounds

General procedure

The mixture of the corresponding dicarboxylic acid (0.01 mol) in 50 cm^3 chloroform and oxalyl chloride (0.05 mol) was stirred and refluxed for



Scheme 1

TABLE 1 Numbers, abbreviations, MS data, and activity of the model compounds

Number	Abbreviation	MS	Hydrolysis of oxindole	Hydrolysis of the substrate
1	*o*-FUM	354	−	−
2	*m*-FUM	354	+	+
3	*o*-DIM	384	−	−
4	*m*-DIM	384	+	−
5	*o*-IPH	404	−	−
6	*m*-IPH	404	−	−
7	*o*-TPH	404	−	−
8	*m*-TPH	404	+	−
9	*p*-TPH	404	−	−

24 h. From the solution the solvent was evaporated under reduced pressure, and the resulting dichloride was dissolved in 50 cm^3 dioxane. Then the corresponding *ortho*-, *meta*-, or *para*-aminobenzoic acid (0.04 mol) was added. The mixture was refluxed for about 24 h. Dioxane was evaporated under reduced pressure, and 200 cm^3 water was added to the residue. The suspension was stirred for about 0.5 h and filtered, washed three times with 50 cm^3 water, and dried. The crystallization from ethanol gives compounds 1–9 as solids. The analytical data and the ^{1}H NMR spectra of all compounds were correct.

Synthesis of the monosalts of compounds 1–9

To the suspension of 10^{-3} mol of compounds 1–9 in 2 cm^3 ethanol was added 1 cm^3 of 1 mol dm^{-3} tetrabutylammonium hydroxide in methanol. The alcohol was evaporated under reduced pressure, and the corresponding salts were dried under reduced pressure in a nitrogen atmosphere.

Complexes of the monosalts of compounds 1–9 with oxindole

The dried monosalts of compounds 1–9 (0.1 mol) were dissolved in absolute ethanol, and 0.1 mol oxindole (Eq. 1) was added. Ethanol was evaporated to prepare the samples for the IR investigation in a carefully dried glove box under a nitrogen stream.

Complexes of the monosalts of compounds 1–9 with oxindole and water

The dried monosalts of compounds 1–9 (0.1 mol) were dissolved in absolute ethanol. To these solutions water (0.1 mol) and oxindole (0.1 mol) were added. The mixture was heated for 10 min at 40°C, and the ethanol was evaporated on the internal reflection element under a nitrogen stream.

Infrared spectroscopy

The spectra were recorded with a Bruker IFS 113 v FTIR spectrometer, using a MCT detector and a resolution of 2 cm^{-1}. As an internal reflection element, a germanium crystal (52 × 18 × 2 mm) with an aperture angle of 45° was used. Forty milligrams of the corresponding compounds were dissolved in ethanol. The ethanolic solutions were spread and dried on this internal reflection element under a stream of dried nitrogen.

Assay for proteolytic activity

The synthetic chromophoric substrate Pro-Thr-Glu-Phe-Phe(NO$_2$)-Arg-Leu (Dunn et al., 1986) was used to determine the activity of porcine pepsin and of the monosalts of model compounds 1–9. The activity of pepsin was assayed at pH 3. In the case of the monosalts the activity was measured after the substances were dissolved in water (pH ~7). The corresponding solutions were added to the solution of substrate (10^{-4} mol dm^{-3}). After the reaction between the model substance and the substrate, the pH value was changed to pH 3 (with diluted HCl), and a water solution of pepsin was added (5 μl, 10^{-3} mol dm^{-3}) to proof the complete splitting of the substrate. The activity was measured with a Perkin-Elmer Lambda UV/Vis spectrophotometer, using the absorption of the chromophor at 300 μm.

RESULTS AND DISCUSSION

All synthesized substances are summarized in Scheme 1 and Table 1. All nine of these substances are *ortho*-, *meta*-, and *para*-aminobenzoic acid diamides of dicarboxylic acids. From this group of substances the monosalt of compound 2 (*m*-FUM), i.e., the monosalt of the *meta*-aminobenzoic acid diamide of fumaric acid, shows catalytic activity that is

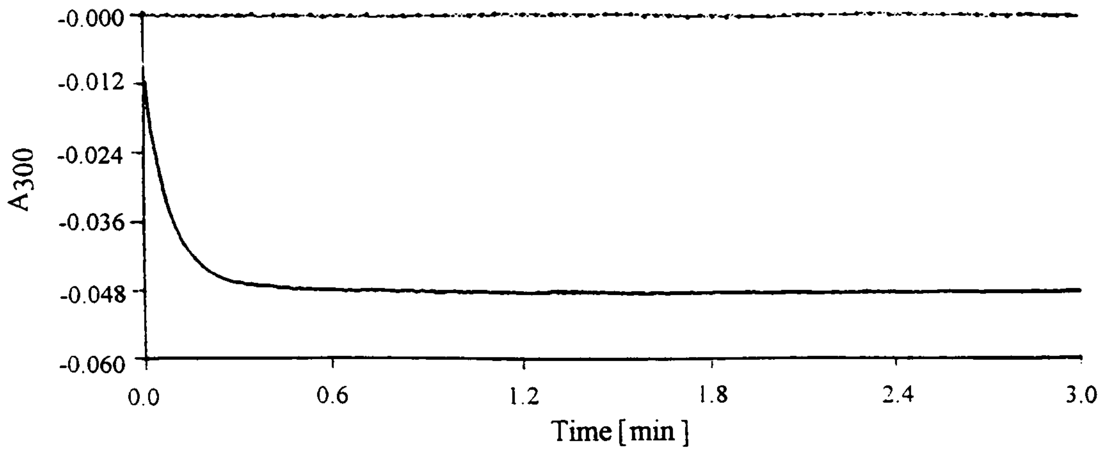


FIGURE 1 The time dependence of the peptide bond splitting of a pepsin substrate by the monosalt of *m*-FUM, measured by the UV band at 300 nm at 298°K (——). The substrate solution without m-FUM (·····).

comparable to the catalytic activity of pepsin. Therefore this substance will be discussed first in more detail.

Discussion of the biological activity of the model molecule *m*-FUM

Fig. 1 proves that *m*-FUM shows catalytic activity with regard to peptide bond splitting with the well-known pepsin substrate Pro-Thr-Glu-Phe-Phe(4-NO_2)-Arg-Leu. This figure shows that if substrate is added to an aqueous solution of the tetrabutylammonium monosalt of compound 2, the absorption at 300 nm decreases strongly, indicating the splitting of the peptide bonds. After about 20 s the absorbance becomes constant. If pepsin is added to the solution of the model molecule with pH 3, no further splitting of peptide bonds is observed. Thus the model molecule has split the peptide bonds that are also split by pepsin, i.e., the Phe-Phe(4-NO_2) bonds.

In Fig. 2 the FTIR difference spectrum of [substrate + pepsin] minus [substrate] (*dashed line*) is compared with the FTIR difference spectrum of the [substrate + the monosalt of the *meta*-aminobenzoic acid diamide of fumaric acid (*m*-FUM)] minus [substrate] (*solid line*). The two FTIR difference spectra are very similar. At 3278 cm^{-1} a negative band is observed. This band is the ν(N-H) vibration (amide A band) of the NH group in the peptide bond. The fact that this band is observed as a negative band demonstrates that with pepsin, as well as with *m*-FUM, peptide bonds are split. This result is confirmed by a very strong negative band at 1629 cm^{-1} and a negative band at 1542 cm^{-1} (Fig. 2 *b*). These bands are the amide I and amide II bands of the peptide group and indicate that the peptide is present before splitting in the β structure. Hence the peptide is refolded. The fact that these bands are observed as negative bands with the same intensity in both cases demonstrates that the peptide bond cleavage by the monosalt of *m*-FUM occurs in the same way as that of pepsin. (The positive bands in Fig. 2 *a* (*solid line*) are the bands of the tetraalkylammonium ions.)

Bands at 1580 cm^{-1} and 1395 cm^{-1} are observed as positive. These bands are, respectively, the antisymmetrical and symmetrical stretching vibrations of the CO_2^- groups in the reaction products.

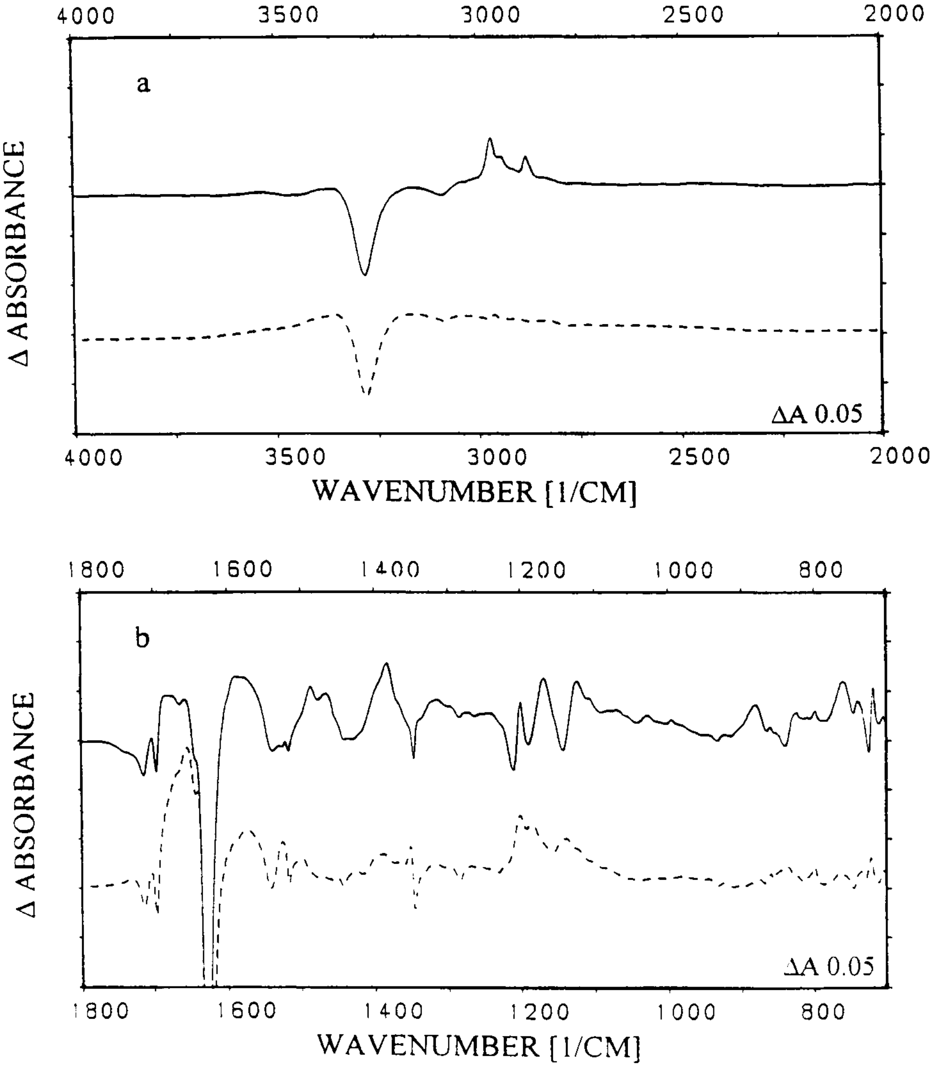


FIGURE 2 FTIR difference spectra of [substrate + monosalt of *m*-FUM] minus [substrate] (——), and, for comparison, [substrate + pepsin] minus [substrate] (– – –). (*a*) In the region 4000–2000 cm^{-1}. (*b*) in the region 1800–700 cm^{-1}

Role of water in peptide bond splitting

We studied the role of water in this peptide bond splitting. Pepsin, as well as the peptide substrate, is not soluble in water-free media. Therefore we used absolute ethanol for this FTIR study. In this solvent *m*-FUM is soluble enough. Furthermore, oxindole is also soluble in absolute ethanol. Oxindole is a lactam, and the amide bond has *cis* configuration.

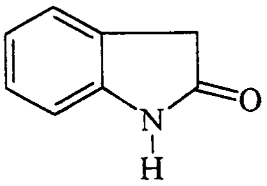
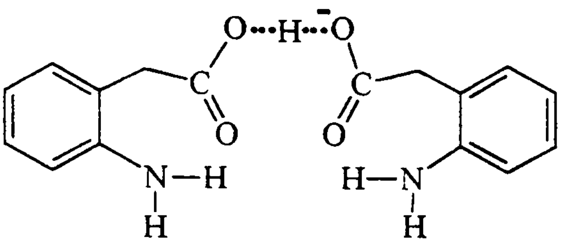


Fig. 3 shows the spectrum of a completely dry 1:1 mixture of the monosalt of *m*-FUM and oxindole (*solid line*) and the spectrum of oxindole (*dashed line*). The comparison of these spectra shows that the ν (C═O) vibration of oxindole shifts from 1710 cm^{-1} to 1692 cm^{-1}. This shift demonstrates that oxindole is bound via a strong hydrogen bond to the carboxylic acid group of the monosalt of *m*-FUM. The other bands of oxindole (e.g., the band at 1386 cm^{-1}) show only small shifts, if any. All of these results show that if no water is present in the solution, the oxindole is strongly bound via a hydrogen bond to the carboxylic OH group of the *m*-FUM monosalt. It is, however, not hydrolyzed.

Fig. 4 shows the spectrum of the 1:1:1 mixture of the monosalt of *m*-FUM, oxindole, and water (*solid line*). Furthermore, it shows the spectrum of a 1:1 mixture of *ortho*-aminophenylacetic acid and the salt of this compound (*dashed line*).

Again, for comparison the spectrum of pure oxindole (*dotted line*) is given:



The comparison of the spectra of the reaction mixture (*solid line*) with the spectrum of oxindole (*dotted line*) shows that all oxindole bands have completely vanished in the mixture. Thus oxindole is completely hydrolyzed.

With regard to the reaction products in the spectrum of the reaction mixture (Fig. 4, *solid line*), an intense IR continuous absorption is most significant beginning at 1600 cm^{-1} and extending toward smaller wavenumbers. Such a continuum indicates homoconjugated carboxylic acid-carboxylate hydrogen bonds (Kristof and Zundel, 1980). In these hydrogen bonds a broad, flat, single-minimum proton potential is present in which the proton fluctuates. These bonds show large proton polarizability, and the continuum arises because of the strong interaction of these bonds with their environments (Zundel, 1976). In these bonds the CO_2-group of *m*-FUM is not involved because the comparison of the spectra drawn with solid lines in Figs. 3 and 4 shows that ν_{as} and ν_s, respectively, of the -CO_2- groups of *m*-FUM are observed in the same positions. In the spectrum drawn with dashed lines in Fig. 4 an intense IR continuum is also observed, indicating a large proton polarizability of the hydrogen bond shown in Eq. 2, as expected. Hence such dimers are also formed in the reaction mixture.

We have discussed the observation that in the complex without water the oxindole molecule is bound by a strong hydrogen bond to the carboxylic acid group of *m*-FUM. If one water molecule is added, the strong field of the carbox-

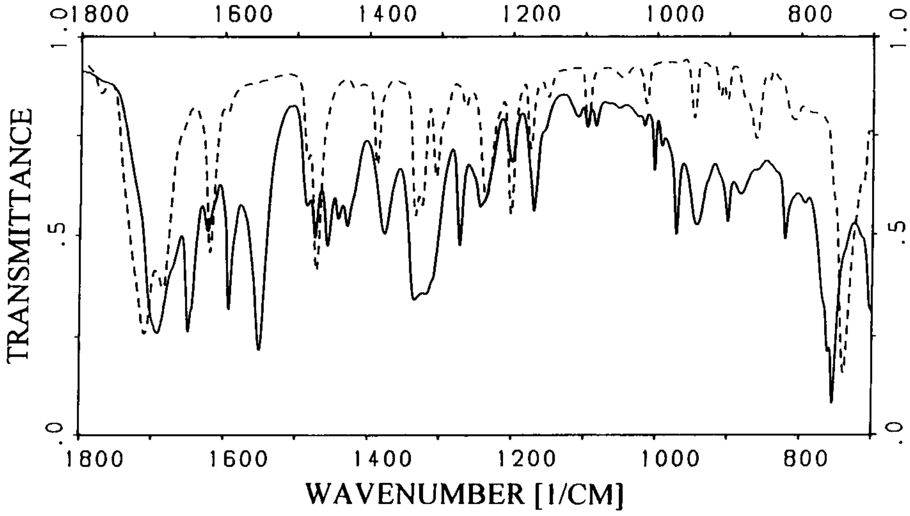


FIGURE 3 FTIR spectra of the monosalt of the 1:1 complex of the monosalt of *m*-FUM with oxindole without water (——) and, for comparison, the spectrum of oxindole (– – –).

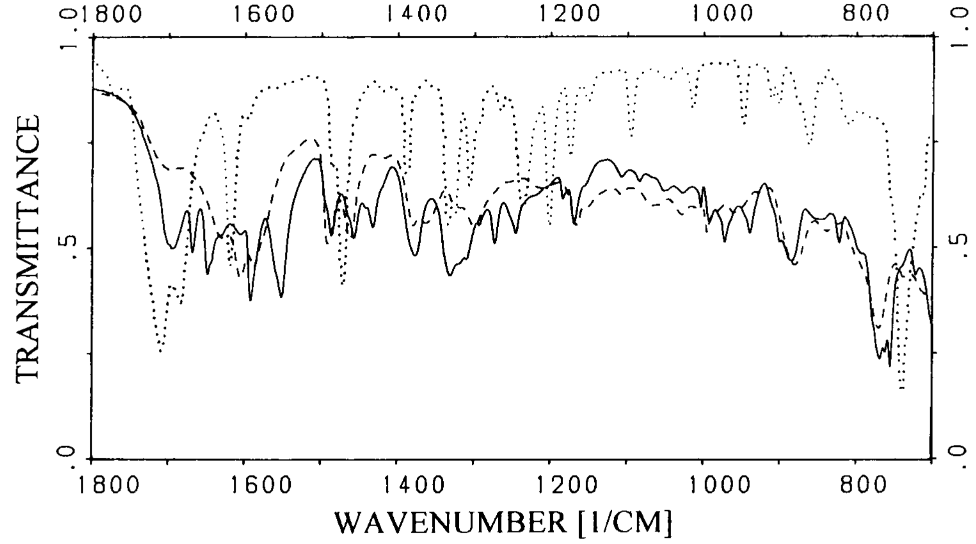


FIGURE 4 FTIR spectra of the 1:1:1 mixture of the monosalt of *m*-FUM, oxindole, and water (——) and, for comparison, the spectrum of a 1:1 mixture of *ortho*-aminophenylacetic acid and the salt of this acid (– – –) and of pure oxindole (·····).

ylate group distorts the water molecule. The negative partial charge of the O atom of this water molecule attacks the electrophilic carbon atom of the peptide bond (Scheme 2). The resulting $(OH)^-$ of the split water molecule is added to the carbon atom of the peptide bond and the proton to the nitrogen. The amide bond is simultaneously broken.

All of these results taken together show that the catalytic mechanism of aspartic proteinases is a base catalysis performed by a distorted water molecule, as postulated by Suguna et al. (1987), James et al. (1992), and Iliadis et al. (1994).

Discussion of the other studied molecules

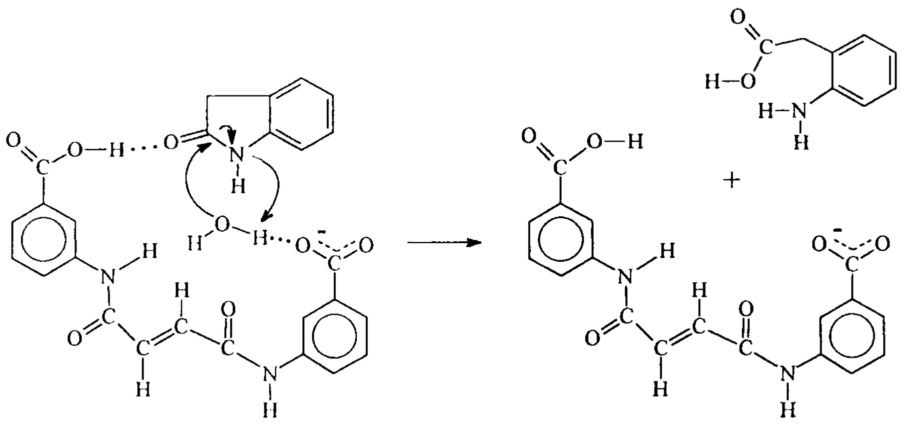
Compounds 4 (*m*-DIM) and 8 (*m*-TPH) also show catalytic activity with oxindole, as shown in Fig. 5, *a* and *b*. The oxindole bands vanish completely, demonstrating that the oxindole molecule is also hydrolyzed by compounds 4 and 8. Furthermore, with these compounds an intense IR continuum is observed, indicating that the above-mentioned reaction proceeds and homoconjugated carboxylic acid-carboxylate hydrogen bonds are formed, showing large proton polarizability.

In water these compounds show, however, no catalytic activity, because the monosalts build disalts and free acids that are completely nonsoluble.

Compounds 1, 3, 5, 6, 7, and 9 show no catalytic activity. There are two reasons for this (see Scheme 1 and Table 1):

If the carboxylic group is in the *ortho* position to the amide group (compounds 1, 3, and 7), an intramolecular hydrogen bond is formed. Therefore, no complexes with oxindole are built, as demonstrated by the ν (C═O) vibration of oxindole. This band is still observed in the mixtures with these compounds at 1710 cm^{-1} and is not shifted toward smaller wavenumbers. This observation is unaffected by the absence or presence of water.

There are steric reasons why compounds 5, 6, and 9 show no catalytic activity. In compounds 5 and 6 the distance



Scheme 2

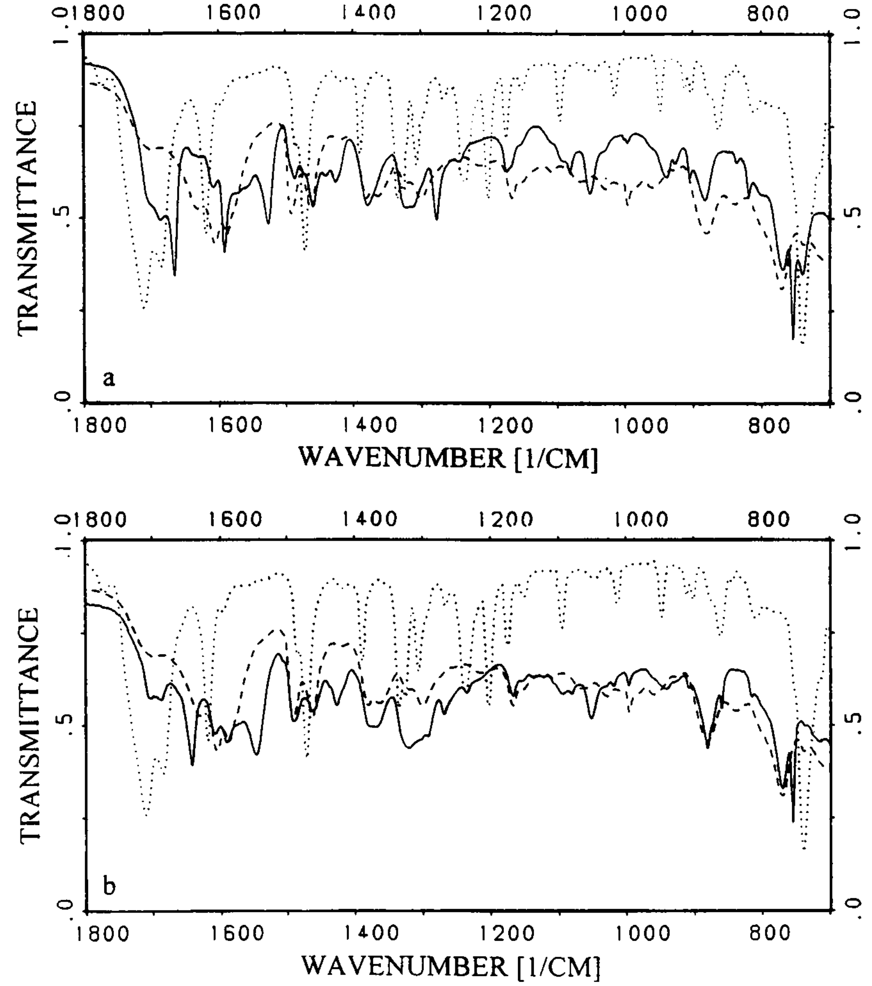


FIGURE 5 FTIR spectra of (*a*) the 1:1:1 mixture of the monosalt of compound 4 (*m*-DIM), oxindole, and water (——) and, for comparison, that of the homoconjugated hydrogen-bonded *ortho*-aminophenylacetic acid with ist acetate (- - -) and of oxindole (·····). (*b*) The 1:1:1 mixture of the monosalt of compound 8 (*m*-TPH), oxindole, and water (——) and, for comparison, that of the homoconjugated hydrogen-bonded *ortho*-aminophenylacetic acid with its respective acetate (- - -) and of oxindole (·····).

between the carboxylic group and the carboxylate group is too small, and in compound 9 it is too long.

CONCLUSIONS

Of nine monosalts of diamides studied, only the monosalt of *meta*-aminobenzoic acid diamide of fumaric acid (*m*-FUM) shows in water the same catalytic activity as pepsin. This result was obtained using a well-known pepsin substrate. This compound also hydrolyzes the amide bond of oxindole if a water molecule is present.

The *m*-FUM forms a complex with oxindole. Herewith the carboxylic group of the monosalt of *m*-FUM forms a relatively strong hydrogen bond to the O atom of the C═O group of oxindole. If one water molecule is added to the complex, the peptide bond is hydrolyzed. The water molecule is strongly distorted because it is hydrogen bonded by a strong hydrogen bond formed with the carboxylate group of the monosalt of the *m*-FUM molecule. The water molecule attacks with its lone electron pairs the electrophilic C atom of the peptide bond. Simultaneously, it is split and the $(OH)^-$ hydroxyl ion is added to the C atom, the C-N bond is broken, and the proton transfers to the N atom.

Thus the catalytic mechanism of aspartic proteinases is a base catalysis, as postulated by James and Sielecki (1985), Suguna et al. (1987), and Iliadis et al. (1994). In this way the carboxylic acid group is bound to the O atom of the peptide bond and the water molecule bound to the carboxylate group is activated. The most interesting result of this study is the fact that a carboxylic acid and a carboxylate group in suitable sterical arrangement are sufficient for the catalytic mechanism of aspartic proteinase if a water molecule is present.

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