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N-Acyl substituted 7-amino-4-chloroisocoumarin: A peptide degradation model via an imide mechanism

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Abstract—During the coupling reaction between 3-alkoxy-7-amino-4-chloroisocoumarin and *N*-acyl alanine dipeptide, an unexpected deamidation reaction was observed. The proposed mechanism for this reaction involved the formation of an imide intermediate which after cleavage led to the release of amino acid moiety. The described deamidation reaction represents the first chemical model involving a non-peptidic moiety, which mimics biological and chemical deamidation processes occurring in proteins or peptides incorporating an asparagine or a glutamine residue.

We have recently reported the remarkable chemical reactivity of 3-alkoxy-7-amino-4-chloroisocoumarin derivatives **1** towards amines[1] as well as their biological activity to inhibit the secretion of β-amyloid peptide,[2] a major component of the senile plaques involved in the Alzheimer's disease. These chemical and biological activities of such isocoumarin derivatives are related to their ability to react with various nucleophiles through a quinonimine methide intermediates.[3]

Continuing our efforts to develop new 4-chloroisocoumarin derivatives of interest for Alzheimer's disease therapy, we investigated the feasibility to couple various specific pseudopeptides at the free 7-amino position of the isocoumarin nucleus using standard DCC/HOBt peptide coupling methodology. *N*-(Difluorophenylacetyl)-alanyl peptide moiety was selected to be coupled to the 7-amino-4-chloroisocoumarin **1**. Indeed, it has been reported that *N*-(3,5-difluorophenylacetyl)-alanine **2** linked to various aromatic heterocyclic nuclei, led to potent β-amyloid peptide inhibitors.[4,5]

Besides their use in the design of β-amyloid peptide inhibitors, the remarkable chemical reactivity of 4-chloroisocoumarin derivatives could also be used in protein deamidation model. Indeed, in vivo protein deamidation processes have drastic consequences in regulatory signal like apoptosis.[6] In this paper, we report a simple chemical model which mimicks well-known in vivo protein deamidation processes.

When 7-amino-4-chloroisocoumarin **1** ($R = OCH_3$) was added to *N*-(3,5-difluorophenylacetyl)-alanine **2** preactivated with DCC/HOBt in the presence of diisopropylethylamine, after standard work up (5% citric acid followed by 5% $NaHCO_3$ washing solutions), LC/MS analysis of the obtained crude mixture revealed the presence of several reaction products represented on Scheme 1.

Besides the expected di-adduct **3**, characterized by LC/MS (peak at $(M+H)^+ = 495$, (retention time) $t_r = 16.90$ min), two other molecular peak ions, that are $(M+H)^+ = 424$, $t_r = 24.40$ min and $(M+H)^+ = 341$, $t_r = 10.49$ min, corresponding respectively to compounds **4** and **5** were identified. These results were repeatedly obtained three times. Compounds **3** and **4** were isolated (19% and 11% yields respectively) upon column chromatography, fully and unambiguously characterized by NMR and LC/MS.[7] Compound **5**, only present as traces (<1%), was identified by LC/MS but not isolated. Besides, starting material **1** was recovered and the overall yield of this uncomplete reaction was 30%.

Keywords: Isocoumarin derivatives; Deamidation reaction; Imide intermediate.
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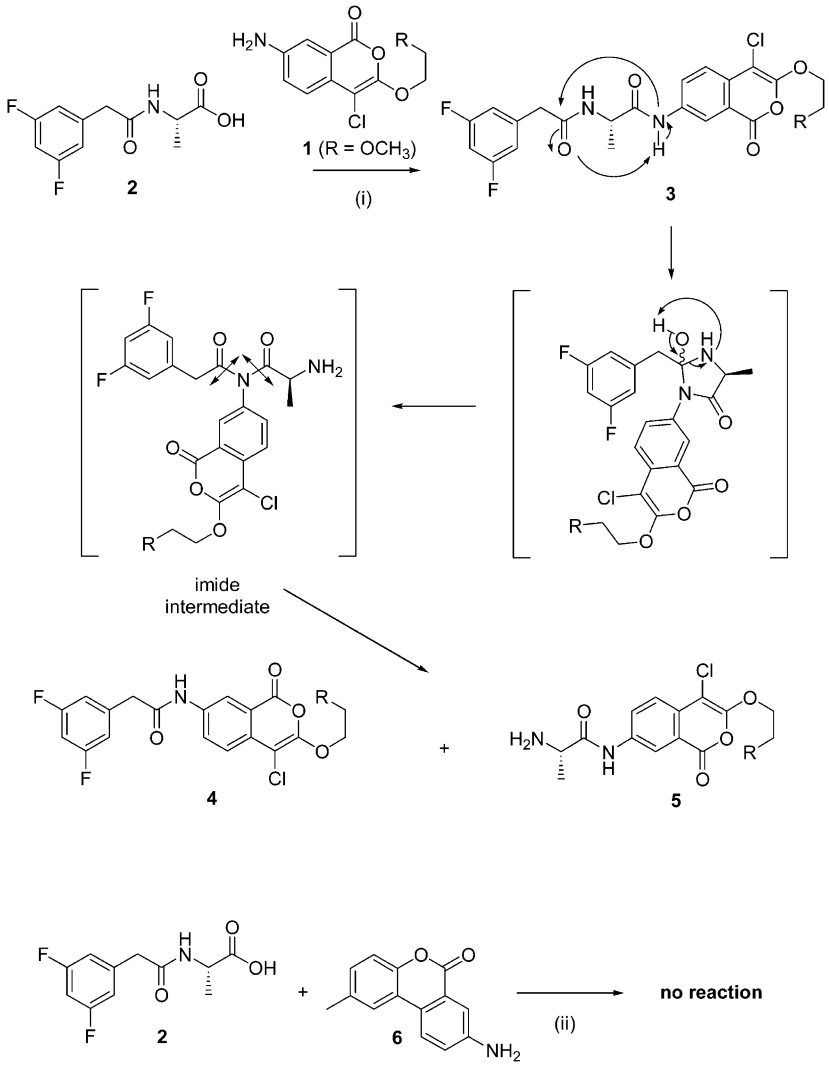


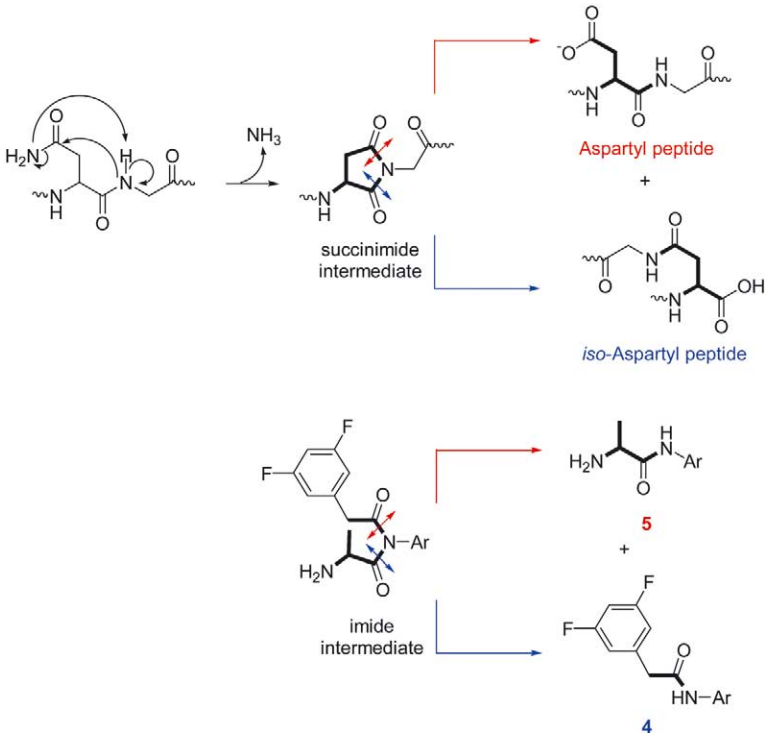
Scheme 1. (i) DCC/HOBt/DiEA, overall yield: 30%; (ii) BOP/DiEA or DCC/HOBt/DiEA.

UV analysis of derivatives **3** and **4** confirmed the integrity of the lactone moiety in each isolated compound. Indeed, no ring opening reaction of the isocoumarin nucleus was observed, as far as compounds **3** and **4** displayed UV absorption spectra (λ max = 380 nm) characteristic of the isocoumarin nucleus.

Moreover analysis of the work up resulting aqueous layer showed the presence of the residue alanine (identified by ninhydrin TLC comparison with a genuine alanine sample). These results led us to postulate a possible mechanism supporting the above observations (Scheme 1). In these experimental conditions, the nitrogen atom at the 7-position of the 4-chloroisocoumarin nucleus of the di-adduct product **3** is sufficiently nucleophile to attack the amide carbonyl group between the difluorophenylacetyl and the alanyl residues to form an imide intermediate. Hydrolysis of this intermediate led to mono-adducts **4** and **5**.

We observed that hydrolysis of this imide intermediately occurred preferentially at the free alanyl side, favoring the 7-*N*-(difluorophenylacetyl)-amino-4-chloroisocoumarin adduct **4**, with a 99:1 ratio between the two obtained degradation products **4** and **5**. Such deamidation reaction mechanism via an imide intermediate is in accordance with already extensively reported mechanisms involving succinimide intermediates, mainly in the case of polypeptide models or natural proteins incorporating an asparagine (Asn) or a glutamine (Gln) residue in their sequences.[8–13]

Indeed, among the numerous forms of irreversible chemical degradations of peptides and proteins, such deamidation is one of the most frequently observed phenomenom which induced the inactivation of the molecules.[14] This reaction can occur during synthesis, purification, manipulation or long-term storage. It is catalyzed by nucleophilic species, enzymes, acids or bases, and is under the influence of temperature, pH of the solution or moisture for solid-state stabilized formulated proteins or peptides.[14,15] Mechanism of such chemical deamidation has long-well been understood but even if the degradation entities are commonly characterized, the rapidly hydrolyzed cyclic imide is not observed.[8–10,16,17] The mechanism of this reaction involved the lability of the protons of the side-chain amino group of the asparagines, which initiated the



Scheme 2. Mechanism of the deamidation reaction: (a) involving a succinimide intermediate, ratio *iso*-Asp (blue)/Asp (red) 3:1;[18] (b) involving a supposed imide intermediate, ratio 4 (blue)/5 (red) 99:1; Ar: 4-chloroisocoumarin residue.

intra-molecular reaction resulting in the formation of the mixture of *iso*-aspartate and aspartate residues (Scheme 2(a)). The dominating species is usually the *iso*-aspartyl one.[18]

One can imagine a similar imide intermediate cleavage to explain the observed ration between the two species **4** and **5**, as depicted on Scheme 2(b).

Indeed, one can notice the structural similarities (bold bonds, on Scheme 2) between the succinimide intermediate (Scheme 2(a)) and our supposed imide intermediate (Scheme 2(b)), that is presence of an α-amino acid residue which hydrolysis in the first case will lead to the generation of an *iso*-aspartyl peptide. As previously mentioned, this species is usually the dominating one.[18] In our case, we observed that the resulting ratio between the two possible cleavages is largely in favor of compound **4**, that is elimination of the alanyl residue, which corresponds to the formation of the *iso*-aspartyl peptide, similarly to the results described in the literature (Scheme 2(a)). This can be supported because of the electronic effect of the *N*-acyl moiety (red arrow cleavage) compared to the free amino group (blue arrow cleavage).

In order to support our supposed mechanism described in Scheme 2, we attempted to perform the same coupling reaction between the *N*-(difluorophenylacetyl)-alanine and an isocoumarin derivative **6** (2-methyl-8-amino-benzo[*c*]chromen-6-one) as model, which structure did not include a chlorine atom on its isocoumarin nucleus. In the same experimental conditions (DCC/HOBt) as well as in other standard peptide coupling conditions (BOP), no desired compound was observed. The unchanged starting material was entirely recovered. This result suggests that in the case of this model without chlorine at the 4-position, the nucleophilicity of the NH-group is too low to allow the coupling reaction. In contrast, this result does not allow to conclude unambiguously that the presence of a chlorine atom dramatically influenced the lability of the NH-proton. It can be envisaged that NH-proton lability is related to the activation or desactivation of the carbonyl function through the resonance effect controlled by the substituents present on the ring bearing the NH moiety.

In conclusion, 7-amino-4-chloroisocoumarin moiety represents a highly reactive species which after coupling with an *N*-acyl substituted peptide, induced an internal amide bond degradation via a mechanism involving an imide intermediate. To our knowledge such peptide degradation model involving an internal reactive species (7-amino-4-chloroisocoumarin) and a peptide sequence which does not incorporate asparagine or glutamine residues has never been described before. Work is in progress in order to generalize this original model to various peptide sequences.

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7. (a) Product **3:** yellow solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.31 (bs, 1H, N*H*), 8.27 (d, 1H, $J = 2.2$ Hz, H_8), 7.93 (dd, 1H, $J = 8.7$, 2.2 Hz, H_6), 7.59 (d, 1H, $J = 8.7$ Hz, H_5), 6.85–6.60 (m, 3H, Ar*H*), 5.60 (d, 1H, $J = 6.9$ Hz, N*H* Ala), 4.78–4.63 (m, 1H, C*H*α Ala), 4.50 (m, 2H, OCH_2CH_2), 3.73–3.76 (m, 2H, CH_2OCH_3), 3.61 (s, 2H, CH_2CO), 3.43 (s, 3H, OCH_3), 1.44 (d, 3H, $J = 7.0$ Hz, CH_3β Ala); LC/MS m/z $(M+H)^+ = 495$. (b) Product **4:** yellow solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.05 (bs, 1H, N*H*), 7.55 (d, 1H, $J = 8.7$ Hz, H_5), 7.45 (d, 1H, $J = 3.0$ Hz, H_8), 7.10 (dd, 1H, $J = 8.5$, 2.5 Hz, H_6), 6.90–6.70 (m, 3H, Ar*H*), 4.47–4.42 (m, 2H, OCH_2CH_2), 3.87 (s, 2H, CH_2CO), 3.76–3.74 (m, 2H, CH_2OCH_3), 3.43 (s, 3H, OCH_3); LC/MS m/z $(M+H)^+ = 424$. (c) Product **5:** LC/MS m/z $(M+H)^+ = 341$.
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