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Synthesis of a chlorothalonil peptide conjugate mimicking protein-bound pesticide residues

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Abstract—A strategy for the synthesis of model conjugates resembling protein-bound pesticide residues was developed on the instance of the fungicide chlorothalonil. Starting from a synthetic dodecapeptide with Fmoc and ivDde protecting groups, a multistep procedure was established for the synthesis of a defined structure.

Despite the necessity of the application of pesticides in agriculture, their use can sometimes cause problems, such as pesticide residues on agricultural commodities. These residues are divided into different subclasses for residue analyses. Extractable pesticides and their metabolites do not cause analytical problems but bound residues cannot be analyzed by routine methods. Increasing efforts are now made concerning the quantification of bound residues by different immunochemical techniques. To understand the reactions leading to bound residues and to study their effects in various areas, it is necessary to have appropriate models.[1,2] There has also been much work in studying the effects of pesticide peptide conjugates regarding the interference of the uptake of amino acids by the amino acid carrier system of plants.[3]

As shown recently in the case of the non-systemic fungicide chlorothalonil (tetrachloroisophthalonitrile), proteins are mostly responsible for the formation of bound residues in plant products.[4] Based on these results, we developed a strategy for the synthesis of a model conjugate for protein-bound residues of chlorothalonil.

In a first attempt, an Fmoc lysine chlorothalonil conjugate was synthesized and employed for the peptide synthesis in a synthesizer, but this conjugate has not been integrated during synthesis. After these experiences a peptide with unmodified amino acids was prepared and subsequently incubated with chlorothalonil yielding a disubstituted product. Thus, the resulting peptide was not suitable for further studies. For this reason, a strategy with different protecting groups was developed. The synthesis was carried out on a peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) protected amino acids.[5] The resulting dodecapeptide (**1**) had the following amino acid sequence: Boc-Gly-Ser(*t*-Bu)-Ala-Leu-Gly-Lys(ivDde)-Ala-Phe-Gly-Phe-Ser(*t*-Bu)-Glu(*O*-*t*-Bu)-resin.

For cleaving the ivDde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl) protecting group, a standard protocol[6] was used first. The resulting peak area ratio of the uncleaved peptide and the chlorothalonil derivative of the cleaved peptide was determined by HPLC[7] after cleavage from the resin. The starting conditions were 2% hydrazine in DMF (*N*,*N*-dimethylformamide) (w/w) and a reaction time of 5 min. The peak area ratio (uncleaved peptide:cleaved peptide) was 7.1:1. An increase of the hydrazine concentration to 5% (w/w) and of the reaction time to 10 min resulted in a ration of 1:1. For the next attempt, the same hydrazine concentration but an extended reaction time of 20 min was used and resulted in a satisfying ratio of 1:19. The latter conditions were used for the following synthesis (Scheme 1).

Abbreviations: Boc, *tert*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl; TFA, trifluoroacetic acid.

Keywords: bound residues; chlorothalonil; peptide synthesis.

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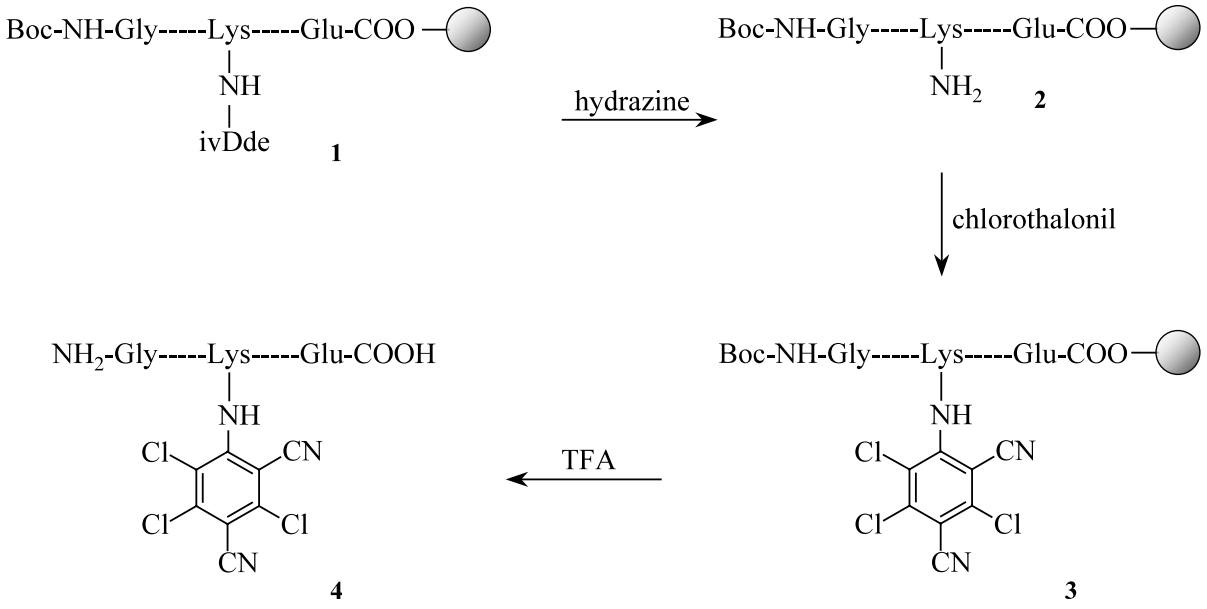

Scheme 1. Synthesis of the chlorothalonil peptide conjugate.

Compound **1** (100 mg; Scheme 1) was placed in a flask and 2.5 mL of a hydrazine solution in DMF (5%, w/w) was added. After 20 min the supernatant was removed and the procedure was repeated twice. The residue (**2**) was put into a glass-filter crucible (G 3) and washed with DMF (10 mL). Chlorothalonil (0.36 mmol, 96.5 mg) was placed in another flask together with acetonitrile (20 mL) and triethylamine (1 mmol, 140 μL), and the peptide (**2**) was added. The mixture was then refluxed for 12 h and filtered through a glass-filter crucible (G 3). The residue (**3**) was cooled on ice and a cooled solution of water (0.5 mL) and TFA (trifluoroacetic acid; 9.5 mL) was added. The ice bath was removed and the solution was stirred at room temperature for 90 min. After filtration, the residue was washed with TFA (1 mL) and methylene chloride (10 mL). The filtrate was concentrated in vacuo and diethyl ether (40 mL) was added while the peptide (**4**) precipitated. It was filtered through a glass-filter crucible (G 4), dissolved in water and lyophilized (yield: 32 mg).[8]

The molecular structure was confirmed by LC–ESI/MS. The peptide ($[M+H]^+$) showed a m/z of 1398.58 (calculated for $C_{61}H_{79}Cl_3N_{15}O_{17}$: 1398.55) with an isotope pattern that exactly matched the calculated pattern.

With this strategy, a defined chlorothalonil peptide conjugate (**4**)[9] was obtained (Fig. 1). Using the ivDde group for the ε-amino moiety of lysine, it was possible to cleave this protecting group selectively without affecting the others. The ivDde group is a hindered variant of the primary amine protecting group Dde (1 - (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidene)ethyl) with the same stability profile, but it does not undergo leaching or side-chain migration.[10] Therefore, it is possible to get defined peptides and reactions with good yields.

In conclusion, this successful strategy can be applied for the synthesis of various pesticide peptide conjugates where the ε-amino group of lysine is used for linking with the active ingredient. These models are necessary and useful for investigations concerning the quantification of bound residues, e.g. by immunoassay, and studying biological effects such as bioavailability or toxicological effects.

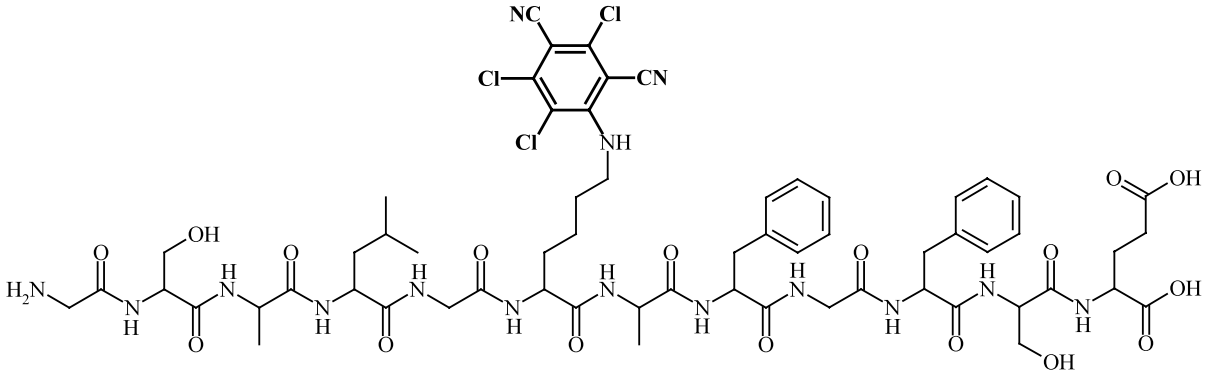

Figure 1. Structure of the chlorothalonil peptide conjugate (**4**).

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7. HPLC conditions: C18 column (3×250 mm; particle size 5 μm); mobile phase: methanol (A) and 20 mM phosphate buffer (pH 4.0) (B) with a gradient program starting from 60% A (5 min) with an increase to 80% A over 7 min, followed by an increase to 100% A over 3 min, a final reduction of A to 60% A over 5 min, held for 5 min; DAD detection wavelength 246 nm.
8. Modified after '*Introduction to Cleavage Techniques*', user manual 343901-002, Applied Biosystems Inc., Foster City, CA, USA.
9. Data for **4**: UV (methanol): λ_{max} [nm]: 248 (log ε=4.2); 303 (log ε=4.1); 358 (log ε=3.6).
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