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An Improved Preparation of a Tertiary Alcohol Proline Linker and its Use in a Synthesis of Mosquito Oostatic Hormone

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Summary: An improved synthesis of 4-(3'-hydroxy-3'-methylbutyl)phenoxyacetic acid was developed using THF to overcome solubility problems initially encountered in trying to reproduce the published preparation. This hydroxyacid linker was coupled to aminomethyl polystyrene resin and used for a synthesis of the mosquito oostatic hormone by the Fmoc protocol. Attempts to prepare this insect peptide by other techniques had failed because of loss of cyclo-Pro_2 from the resin.

In 1985, Borovsky reported the isolation and partial characterization of a material that inhibited vitellogenesis in the mosquito *Aedes aegypti*.[1] The material had the properties of a peptide; the structure was subsequently reported[2,3] as the decapeptide NH_2-Tyr-Asp-Pro-Ala-$(Pro)_6$-COOH. The synthetic hormone, prepared[2,3] by "standard automated solid-phase peptide techniques," was shown to be identical to the natural material. As we wished to test this material and its analogs on other insects in our laboratory, attempts at its synthesis were made. The resulting resin lost weight corresponding to the cleavage of all or nearly all of the initial Boc-Pro attached, leaving an essentially bare resin. Gisin and Merrifield[4] reported that (using Boc protection) only 30% of the expected tripeptide was formed during coupling of D-Pro to D-Val-L-Pro-O-Resin. They studied this loss systematically and determined that it did not occur during the deprotection or neutralization step. Instead, it occurred during the coupling reaction. This was effected by addition of dicyclohexylcarbodiimide (DCC) to a mixture of the dipeptide resin and Boc-D-Pro-OH, and the free carboxylic acid of the protected D-Pro catalyzed the formation of the diketopiperazine cyclo-(D-Val-L-Pro), with the concomitant loss of the peptide chain from the resin. They overcame this by reversing the addition order of the DCC and amino acid, thereby eliminating concentrations of free carboxylic acid. In our first synthetic attempt at the oostatic hormone by the Boc protocol, Boc-Pro-O-Resin (500 mg, 0.36 mmol/g) gave, after the synthesis, only 441 mg of "peptide-resin", an amount which corresponded closely with the calculated weight of the bare resin. Another synthesis by the Fmoc/BOP protocol, with a reduced deblocking period for Pro^9 gave only 490 mg of air-dry peptide-resin from 502 mg of 0.28 mmol/g Fmoc-Pro-O-Resin. The calculated weight of the bare resin was 454 mg, and the cleaved product showed none of the desired peptide by HPLC. Fmoc/BOP synthesis of a shorter segment of the hormone (Tyr-Asp-Pro-Ala-Pro-OH) yielded only 13 mg of cleaved peptide, compared to a theoretical yield of 76 mg, or a crude yield of only 17%.

The "standard methods" that Borovsky *et al.* refer to are those of Barany and Merrifield.[5] The techniques mentioned in this reference for overcoming diketopiperazine formation are: (a) preactivation or addition of the amino acid to a slurry of the dipeptide-resin and DCC, (b) *in situ* neutralization of a salt of the dipeptide-resin with the N-methylmorpholine salt of the protected amino acid in the presence of the condensing agent, and (c) coupling a tripeptide to the resin or coupling a dipeptide to the initial amino-acid resin, bypassing the dipeptide-resin stage. Both the tBoc and Fmoc protocols used with our Milligen/Biosearch 9600 involve preactivation. Technique (b) is not applicable in Fmoc syntheses

because a salt is not generated during the deprotection. Therefore, route (c) was used.

A crude preparation of Fmoc-Pro-Pro-OH was made from Fmoc-Pro and Pro-OtBu, followed by deprotection with trifluoroacetic acid. The material could not be crystallized, but when used in a synthesis of the oostatic hormone amide using PAL resin[6] gave 148 mg of crude peptide (85% pure by HPLC) from 500 mg resin. In contrast, synthesis of the acid form of the hormone failed (only 14 mg crude peptide was obtained from 500 mg of resin, the crude peptide-resin had lost 18 mg.) Although it is not known why this technique failed in this case, it has been used successfully in the synthesis of the sweet protein Monellin and some of its analogs.[7-9]

In 1990, Akaji *et al.* reported the use of a tertiary alcohol handle ($HOC(CH_3)_2CH_2CH_2C_6H_3OCH_2COOH$) for proline which increased the hindrance about the linking ester group, thereby inhibiting intramolecular cleavage.[10] A nearly quantitative yield of crude bradykinin potentiator B , having a Pro-Pro sequence at the C-terminal end of the molecule, was obtained. Unfortunately, the description of the synthesis of this linker by Akaji *et al.* was schematic, lacking any details. Our attempts to prepare this linker indicated that the synthesis was very sensitive to solvent. We found that the initial Grignard reaction of 4-(4-hydroxyphenyl)-2-butanone ("raspberry ketone") with methylmagnesium bromide (the published procedure used methyllithium, but in our hands this did not react completely) only went to completion when the reaction was run in tetrahydrofuran as solvent. The phenoxyacetic acid was prepared from the phenol with sodium ethoxide and bromoacetic ester in absolute ethanol. The material could also be prepared in acetone with potassium carbonate. However, this method was very sensitive to particle size of the potassium carbonate, and excess bromoacetic ester made the workup rather unpleasant. Saponification completed the synthesis of the linker. Our overall yield was 83% compared to the 60% reported by Akaji *et al.*

Aminomethyl resin was coupled to the linker with diisopropylcarbodiimide in dichloromethane/DMF, then coupled to Fmoc-Pro-Cl in pyridine/dichloromethane as reported.[10] Elongation of the chain using the Fmoc-BOP protocol gave, after cleavage, 103 mg of crude peptide (76% pure) from 500 mg of Pro-resin. The HPLC- purified peptide had the correct amino acid analysis: Asp 1.0 (theory 1), Ala 1.15 (1), Tyr 1.0 (1), and Pro 6.6 (7). The peptide sequenced Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro... (the repetitive yields fell off rapidly after Pro^6).

EXPERIMENTAL

Peptide syntheses were carried out on a Milligen/Biosearch 9600 instrument, initially using the manufacturer-supplied protocols. Software adjustments were made as necessary during the various preparations. HPLC separations utilized acetonitrile/water gradients containing 0.05% trifluoroacetic acid on a YMC 6x250 mm column of 5-micron 300A pore size C_4 reversed-phase packing. Unless otherwise indicated, all solvents were of technical grade, redistilled in glass before use. Peptide sequencing was carried out on an Applied Biosystems Model 477A sequencer.

4-(4-hydroxyphenyl)-2-methyl-2-butanol

To a 3 liter 3-neck round-bottom flask, fitted with a mechanical stirrer, cold-finger condenser, and addition funnel, was added 32 g (1.3 moles) magnesium turnings (Alfa Inorganics, resublimed). Dry THF (see footnote[11]) (Burdick and Jackson, 500 ml) was added, then 80 ml methyl bromide was

distilled in over 2 hours at a rate sufficient to maintain gentle reflux. The dark solution was then heated under reflux for 1 hour. THF (500 ml) was added, followed by 4-(4-hydroxyphenyl)-2-butanone (50 g, 304 mmol, Aldrich) dissolved in enough THF to bring the volume to 400 ml. Addition took 35 minutes. The reaction mixture became viscous and cloudy, but did not gel. No obvious precipitate appeared. The solution was heated under reflux for 1 hour, and the viscosity decreased during this time. Examination of an aliquot by gas chromatography showed only a trace of starting ketone. The reaction mixture was hydrolyzed with saturated aqueous ammonium chloride (about 160 ml was required to give a clean separation of white precipitate) and filtered. Removal of solvent gave 63.6 g of product (white solid) which was used directly in the next step. The crude material can be recrystallized from aqueous methanol to give colorless crystals, mp 128-132.5° C. (Reported mp 134-135° [12], 130-131° [13])

4-(3'-hydroxy-3'-methylbutyl)phenoxyacetic acid

A 3 liter 3-neck round-bottom flask was fitted with a mechanical stirrer, an addition funnel, and a Claisen adapter fitted with a water-cooled condenser and another addition funnel. The crude tertiary alcohol was added, dissolved in 400 ml of absolute alcohol. In one addition funnel was placed a solution of sodium ethoxide, prepared from sodium (16.1 g, 700 mmol) in 350 ml absolute alcohol. In the other funnel was placed ethyl bromoacetate (79 ml, 700 mmol) in sufficient ethanol to make *ca.* 200 ml. The two solutions were added simultaneously, with the NaOEt solution flow rate maintained twice as fast as that of the ester (addition took 2 hr). The solution was then heated under reflux for an additional hour. The solution was diluted with ether, washed thrice with water and dried over $MgSO_4$. Removal of solvent on a rotary evaporator and evacuation at oil-pump vacuum (<1 mm) left 101 g of a yellow oil which could not be crystallized. Dissolution of the oil in ethanol (400 ml) followed by addition of a solution of 38 g KOH in 50 ml water and heating under reflux for an hour saponified the ester. Solvent was removed on a rotary evaporator, followed by evacuation at oil-pump vacuum over the weekend (3 days) to remove the last traces of ethanol. The waxy solid was dissolved in water, and the aqueous solution was extracted with three portions (*ca.* 100 ml each) of ethyl acetate and acidified with HCl (pH ≈1). The crude acid that precipitated was extracted with two 100-ml portions of ethyl acetate, washed once with water, once with dilute brine, once with saturated brine, dried over $MgSO_4$, and evaporated to give a yellow solid (73 g). This was dissolved in methyl t-butyl ether (*ca.* 400 ml), treated with charcoal, then boiled down to a volume of *ca.* 200 ml and cooled to 5°. Filtration gave 57.5 g colorless crystals, mp 104-106° C (Reported[10] 101-102° C). Recrystallization of a small second crop gave an additional 3 g, with the same melting point. The combined product represented an 83% yield overall (see reference[14]). Mass spectrometric examination of the purified product as its methyl ester (diazomethane) showed the molecular ion at m/z 252, and the expected fragment ions.

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11. Other solvents were tried. Raspberry ketone was difficultly soluble in ether, but could be added in warm toluene or methyl t-butyl ether. Addition of ketone to solutions of MeMgBr in ether, ether/toluene, ether/dioxane, and methyl t-butyl ether gave thick gelatinous precipitates or rubbery solids which could not be stirred, as did addition of methyllithium to a solution of ketone in warm ether. Hydrolysis of these products gave mixtures of alcohol and starting ketone (24 - 35%). THF was the only solvent tried that did not present this problem, giving stirrable solutions and crude product containing only traces (<1%) of starting material. If preparation of MeMgBr is not desired, MeMgCl in THF is available from Aldrich. Examination of the tertiary alcohol by GC was hampered by slight decomposition (ca. 5%) in the injector port to give two products eluting earlier than the alcohol. Similar early-eluting peaks were seen in the GC trace from the phenoxyacetic acid, corresponding to those from a sample of the phenoxyacetic acid which was accidentally overheated on a hot plate during recrystallization. This mixture gave a single product on hydrogenation, presumably *p*-isoamylphenoxyacetic acid. This decomposition may also be responsible for the wide melting point range of the alcohol, although there was no obvious decomposition at the melting point.
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14. Crystallization could be carried out less conveniently from aqueous ethanol or aqueous acetone (the product tended to oil out, and the recovery was lower). If small crystals are desired for their quick-dissolving properties, the warm solution in methyl t-butyl ether should be seeded and stirred while the initial rapid crystallization occurs, then periodically until separation of product is complete.

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