Natural and Synthetic Materials with Insect Hormone Activity


KAREL PODUSKA and FRANTIŠEK SORM

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague

KAREL SLAMA

Institute of Entomology, Czechoslovak Academy of Sciences, Prague

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Systematic alterations of the central amino acid in the peptidic juvenile hormone analogues consisting of three parts connected by amidic bonds tert.-butyloxycarbonyl group — amino acid — p-aminobenzoic acid ethylester) have revealed that the hormonal activity is dependent on the 1-configuration of the central amino acids. The compounds exhibit specific activity on insects of the family Pyrrhocoridae, being inactive on several other insects tested.

In the foregoing paper 1 we have described new analogues of insect juvenile hormone (JH), which consist of three parts connected by amidic bonds. The most active JH analogue has appeared to be a tripeptide derivative ethyl L-isoleucyl-L-alanyl-p-aminobenzoate (I).

The results presented in the paper 1 allow to deduce that the L-isoleucine residue in the structure 1 with a free amino group may not be essential for the biological activity; replacement of the amino group by chlorine atom has not been followed by a change of activity.

We have now discovered that the mentioned L-isoleucine residue can be successfully replaced by a tert.-butyloxycarbonyl group (Boc), which is, like isoleucine, characterized by a bulky aliphatic...
chain. The Boc-group contains a quaternary C-atom important for JH activity of various type JH analogues and, in addition, the amino acids bearing this group are currently used for peptide syntheses. In this study we have been altering the central part of the molecule analysing relationships between structure and JH activity. This central part has been represented by various amino acids substituted always at the amino group by the mentioned Boc-group, and with the carboxyl connected to the ethyl p-aminobenzoate.

Methods

The compounds presented in Fig. 1 were prepared by condensation of Boc-derivatives of L-, D-, or DL-amino acids with the ethyl p-aminobenzoate. The starting Boc-amino acids were obtained by the pH controlled reaction of tert-butylazidoformiate with the corresponding amino acids. The condensations were performed either according to a phosphazomethod (method A), or by using dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole (method B).

The exact reaction conditions are as follows:

1. The JH activity was assayed on freshly molted last instar larvae of *Pyrrhocoris apterus* L., *Dysdercus cingulatus* (Fabr.) - *Pyrrhocoridae*; *Graphosoma italicum* Müll. - Pentatomidae; and on freshly molted pupae of *Tenebrio molitor* L. - *Tenebrionidae*. The compounds were applied topically in a constant 1 μl drop of acetone solution or injected in 1 μl of olive oil suspensions. The JH activity was evaluated according to the inhibition of metamorphosis after the next molt.

2. The values presented in Table I are expressed in standard JH activity units (ID-50), indicating the amount of a substance in μg per specimen which caused, under the above mentioned conditions of application, exactly half-larval or half-pupal adultoids.

**Results and Discussion**

The assay results allow to make certain conclusions concerning the optimal structure of the central amino acid. It appears that the most important factor determining the activity is the sterical arrangement of the whole molecule. And, as a matter of fact, the sterical arrangement of our compounds is mostly influenced by the sequence of the substituents around the optically active C-atom located in the central amino acid. This is manifested by differences in JH activity between two pairs of optical antipods (2-D, 3-D) and (2-2-D, 3-2-D). The L-alanine(2) and L-proline(3) derivatives were both very active, whereas their optical antipods (2-D, 3-D) containing the corresponding D-amino acids were almost inactive (Table I). However, it is possible that these derivatives containing D-amino acids were actually completely inactive, and their very low activity was just due to the presence of trace amounts of the corresponding active L-forms.

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7 Abbreviations of amino acid derivatives and peptides according to European J. Biochem. 1, 375 [1967].
9 Method A: The solution of ethyl p-aminobenzoate (1.65 g) in dry pyridine (25 ml) is mixed with freshly distilled PCl₅ (0.45 ml) at -20 °C and allowed to stay for 30 min at -20 °C and 30 min at room temperature. Then, after addition of the corresponding Boc-amino acid (8.5 mmoles) the reaction mixture is refluxed for 3 hrs. After evaporation of pyridine the residue is dissolved in ethylacetate (50 ml) and washed twice (2 x 10 ml) with water, 5 per cent citric acid, water, 5 per cent sodium bicarbonate, water, and finally dried over sodium sulphate. The ethylacetate is then evaporated under vacuum and the residue recrystallized.
10 Method B: The solution of Boc-amino acid (9.5 mmoles) in dimethylformamide (DMF) (10 ml) is mixed at -7 °C with 1-hydroxybenzotriazole (1.5 g) and a solution of dicyclohexylcarbodiimide (2.3 g) in DMF (7 ml). It is then allowed to react 1 hr at -7 °C and 1 hr at 20°C. This reaction mixture is then cooled to 0 °C, mixed with the solution of ethyl p-aminobenzoate (1.65 g) in DMF (5 ml) and allowed to react for 24 hrs at the same temperature. The precipitate of dicyclohexylurea is filtered out, the filtrate is evaporated under vacuum and the dry residue is worked up as under the Method A.
<table>
<thead>
<tr>
<th>Compounda</th>
<th>Central amino acidb</th>
<th>Method of preparation</th>
<th>M. p. [°C]</th>
<th>[α]D&lt;sup&gt;c&lt;/sup&gt; (c 0.5 in DMF&lt;sup&gt;e&lt;/sup&gt;)</th>
<th>ID-50 Units of JH activity&lt;sup&gt;d&lt;/sup&gt; on:</th>
<th></th>
</tr>
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<tr>
<td>2</td>
<td>Ala</td>
<td>B</td>
<td>126—128</td>
<td>−16.4&lt;sup&gt;°&lt;/sup&gt;</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>2 D</td>
<td>D-Ala</td>
<td>B</td>
<td>127—129</td>
<td>+15.6&lt;sup&gt;°&lt;/sup&gt;</td>
<td>300</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>Pro</td>
<td>B</td>
<td>164—166</td>
<td>−41.8&lt;sup&gt;°&lt;/sup&gt;</td>
<td>0.09</td>
<td>0.08</td>
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<tr>
<td>3 D</td>
<td>D-Pro</td>
<td>A</td>
<td>165—167</td>
<td>+42.0&lt;sup&gt;°&lt;/sup&gt;</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>4</td>
<td>Gly</td>
<td>B</td>
<td>79—81</td>
<td></td>
<td>&gt;500</td>
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<tr>
<td>5 DL</td>
<td>DL-Abu</td>
<td>A</td>
<td>156—158</td>
<td></td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>6</td>
<td>Ile</td>
<td>B</td>
<td>128—130</td>
<td>+14.6&lt;sup&gt;°&lt;/sup&gt;</td>
<td>0.05</td>
<td>0.01</td>
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<tr>
<td>7</td>
<td>Phe</td>
<td>A</td>
<td>140—142</td>
<td>+54.9&lt;sup&gt;°&lt;/sup&gt;</td>
<td>&gt;500</td>
<td>500</td>
</tr>
<tr>
<td>8 DL</td>
<td>D-β-Abu</td>
<td>B</td>
<td>162—164</td>
<td></td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Sar</td>
<td>A</td>
<td>160—162</td>
<td></td>
<td>300</td>
<td>&gt;500</td>
</tr>
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</table>

Table I. Chemical characteristics and JH activity of the compounds listed in Fig. 1.

a Elemental composition (C, H, N) corresponded with the structures given. Purity of the compounds was checked by electrophoresis after splitting out the Boc-group by trifluoroacetic acid. b Standard symbols for amino acids are used. All amino acids have L-configuration (except Gly and Sar); the D-forms or DL mixtures are indicated. c Abbreviations: Ea—ethylacetate, Pe—light petroleum, Et—ethanol, W—water, DMF—dimethylformamide. d The activity given in µg per specimen after topical application on uninjured larval cuticle.

Another factor important for JH activity represents branching of the chain in the central part. The glycine derivative 4 without branching (R² = H) has no JH activity, while the methyl-branched alanine derivative (2, R² = CH₃) is very active. The size of the aliphatic branching radical R² does not seem to be decisive. The alanine 2 and α-amino-butyric acid derivative (5 DL) with rather small methyl or ethyl radicals, as well as the isoleucine derivative 6 with relatively large radical [R² = C₃H₆·CH(CH₃)], give all similar JH activity responses. Nevertheless, the JH activity disappears when the branching radical is represented by an aromatic ring (R² = C₆H₅·CH(CH₃)), as documented by the phenylalanine derivative 7.

Further factors which influence the JH activity are determined by the position of the branching radicals R², or R¹, in the chain. The results indicate that a distance of the atom which bears the branching radical from both terminal parts is less important for the activity than the above described steric arrangements. We have been interested to compare the JH activity of three methyl-branched compounds (2, 8 DL, 9) with different positions of the methyl radical. One pair of them, i.e. the alanine derivative 2 and β-aminobutyric acid derivative (8 DL) differ from each other by different distance of the methyl radical from the benzene ring (3, or 4 atoms resp.). Both have high JH activity. On the other hand, the second pair, i.e. the β-aminobutyric acid derivative (8 DL) and sarcosine derivative 9 have both the methyl radical located on the 4th atom from the benzene ring, however, the methyl radical in the former is located on the C-atom, whereas that of the latter represents the R¹ radical on the N-atom; the sarcosine derivative 9 has no JH activity. Such inactivity cannot be explained only by the presence of branching on the N-atom, because the biologically active proline derivative 3 is also branched on the N-atom. It is more likely that the reason for inactivity of the sarcosine derivative 9 would depend in the absence of branching on the C-atom, similarly like it is the case in the biologically inactive glycine derivative 4. It is necessary to point
out that the DL compounds 5, 8 were prepared from DL-aminobutyric acids. By analogy with the compounds 2 – 2 D and 3 – 3 D we expect that the biologically active components of the racemates were only their L-forms.

All the compounds listed in Table I were subjected to additional topical or injection bioassays on a Pentatomid bug Graphosoma and on pupae of Tenebrio. No JH activity was recorded here and also in other orientation tests performed on Gryllus bimaculatus, Dermestes vulpinus, Drosophila melanogaster, etc. Evidently, all these compounds are highly specific, acting only on the insects of the family Pyrrhocoridae, similarly like the JH analogues of juvabione type 2. Contrary to the optical isomers of juvabione 8, which all retain the JH activity 2, the compounds we have described are active only when containing the natural L-amino acids. This phenomenon can perhaps be explained by a more rigid structure of the molecules containing amidic bonds.

In order to obtain more information on the specificity and structure-activity relationships of these hormone analogues, we have prepared series of compounds in which other parts of the molecule have systematically been altered. The results will be published later.

Cell Cycle Analysis and Stimulation of Rat Kangaroo Cells (PtK₂) after Pulse Labeling

PETER R. LORENZ * and JOHN W. AINSWORTH
Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., 12201, USA
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The phases of the cell cycle of Potorous tridactylus (PtK₂) cells were determined in vitro by analysis of labeled mitoses for 37-1/2 hours after a tritiated thymidine pulse. The mean duration of DNA synthesis (t₅) was 8 h. The mean duration of the gap phase before appearance of labeled mitoses was 5 h. Whereas the duration of the cell cycle (tₖ) based on analysis of labeled mitoses was 30 h, the doubling time (tₐ) derived from cell counts in the same cultures was only about 23 h. The analysis of the indices of labeled nuclei and mitoses suggests a stimulation of cells at the time of pulse labeling, which was maximal after beginning of the gap phase before DNA-synthesis, and possibly caused the observed difference between tₖ and tₐ.

The cell cycle time (tₖ) of permanent cell lines established from kidneys of the rat kangaroo, Potorous tridactylus has been found to be 28 to 32 h. No data on the phases of the cell cycle of Potorous tridactylus cells have been published. Although the duration of DNA-synthesis (t₅) of mammalian cells was often found to be close to 8 h, significant variations have been described. Since cells of Potorous tridactylus are ideally suited for chromosomal studies, we determined the cell cycle parameters of PtK₂ cells in non-synchronized monolayer cultures by analysis of labeled mitoses. The results were related to the doubling time (tₐ) of the same cultures.

Cells (2.5 × 10⁴ per ml) were suspended in Eagle’s minimum essential medium 8 containing 10 percent fetal bovine serum, and seeded on cover glasses in 6-cm Falcon Petri dishes. After 3 days of incubation in an atmosphere of humidified air containing 5 percent CO₂, this medium was replaced for 10 to 17 min with medium containing 7 × 10⁻⁷ M tritiated thymidine.

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* Present address: Institut für Experimentelle und Klinische Virologie der Deutschen Forschungsgemeinschaft, D-1000 Berlin 42, Wenckebachstraße 23, Germany.
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