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# Activities of novel polyhydroxylated cardiotonic steroids purified from nuchal glands of the snake, *Rhabdophis tigrinus*

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Seven novel polyhydroxylated steroids were isolated from the nucho-dorsal glands of the snake, *Rhabdophis tigrinus*. Biological activities of these steroids in inhibiting $(Na^+ + K^+)$ATPase and in producing positive inotropic action were examined in comparison with those of ouabain and gamabufotalin. Gamabufotalin was approximately 10 times more potent than ouabain in inhibiting $(Na^+ + K^+)$ATPase. Two compounds, compounds III and XIII, of the seven, produced nearly equipotent enzyme inhibitory activity to ouabain. The activity of the remaining five was relatively low among the compounds tested. All compounds exhibited more or less positive inotropic action in the papillary muscle preparations. The ranking order of the potency was: gamabufotalin > ouabain and compound IV > compound III and XIII > compound I, II, XII and XIV.

We have reported (Akizawa et al 1985a, b) that nuchodorsal glands of the snake, *Rhabdophis tigrinus*, contain novel steroids. These are analogues of the known bufodienolides, but differ from them in the polyhydroxylation and configuration of the hydroxy group. It is generally known that bufodienolide toxins obtained from the family of *Bufonidae* possess a potent inhibitory activity on $(Na^+ + K^+)$ATPase and produce a positive inotropic action (Flier et al 1980; Ku et al 1974). However, it is not known whether the newly identified steroids produce an inhibitory effect on $(Na^+ + K^+)$ATPase and a positive inotropic action. Therefore the present experiments were undertaken.

## *Materials and methods*

*Preparation of steroids from nuchal glands.* The preparation of steroids from nuchal glands of *Rhabdophis tigrinus*, was as described by Akizawa et al (1985a, b). In brief, the faint yellowish, sludgy secretion was collected by pressing the glands with forceps and was then lyophilized. A sample of the lyophilizate was extracted with 5% acetonitrile and the extract chromatographed (HPLC: JASCO, Tri Rotar-II) on an ODS column (Hibar Lichrosorb RP-8, 4 × 250 mm, elution; 5 to 50% $CH_3CN$/60 min at linear gradient, flow rate; 1 ml $min^{-1}$, monitored at 300 nm). The purity of each fraction was determined by HPLC, mass spectrometry (JOEL, JMS-D-300) and $^1H$ NMR (JOEL, FX-270). Chemical structures were assigned with the aid of $^{13}C$ NMR (JOEL, FX-270) by comparison with those of structurally known bufodienolides such as gamabufotalin, bufotalin, arenobufagin, cinobufagin and marinobufagin. Seven novel steroid structures (compounds I, II, III, IV, XII, XIII and XIV) were identified. Tentative chemical structures of these steroids are shown in Fig. 1.

*$(Na^+ + K^+)$ATPase (EC 3.6.1.3) assay*

Canine kidney $(Na^+ + K^+)$ATPase was obtained from Sigma (specific activity: 1·6 μmol $min^{-1}$ (mg protein)$^{-1}$). This preparation also contained a ouabain-insensitive $(Mg^{2+})$ATPase which was less than 3% of the total steady-state ATPase activity. $(Na^+ + K^+)$ATPase was prepared for assay by dissolution in the buffer containing 25 mM mannitol, 20 mM Tris-HCl and 1 mM $Na_2EDTA$ (pH 7·4).

Five to seven concentrations of the test solution or vehicle (0·1 ml each) were incubated for 5 min in a shaking water bath at 37 °C in a 0·7 ml reaction media containing (final mM concentration): NaCl 100; KCl 10; $MgCl_2$ 3; $Na_2EDTA$ 0·2; Tris-HCl 3·2 (pH 7·4 at 22 °C). Then, $(Na^+ + K^+)$ATPase solution (0·03 u/0·1 ml) was added. Prolonged incubation caused decreased activity in the absence of ATP. Therefore, the incubation time was exactly 8 min. Reactions were initiated by the addition of γ-labelled [$^{32}P$]ATP (0·15 μCi $ml^{-1}$) to give a final concentration of 1 mM. Total volume of the reaction mixture was 1 ml. The enzyme reaction was stopped with 1 ml of ice-cold 10% TCA solution after 30 min reaction time. The samples were kept on ice for 10 min before 3 ml of TCA solution containing charcoal

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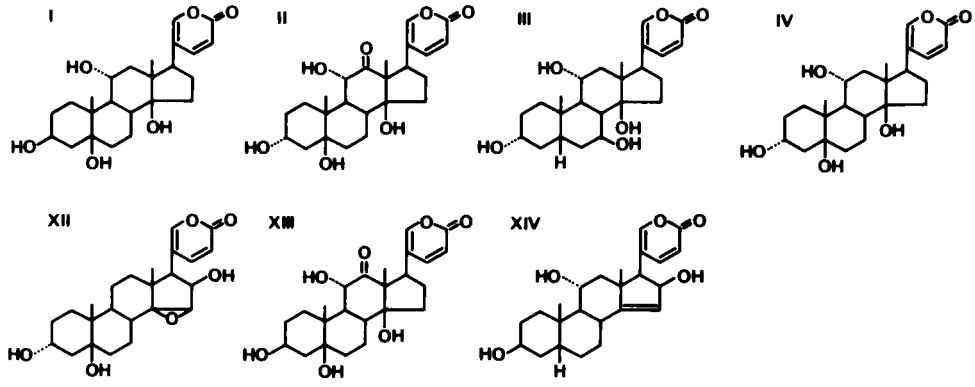


FIG. 1. Tentative chemical structures of novel cardiotonic steroids.

(10% TCA and 20% charcoal) was added to each test tube, mixed, cooled on ice for another 15 min, and then centrifuged at 3000 rev $min^{-1}$ for 5 min. A 0·5 ml aliquot of supernatant fraction was removed and counted in an Aquazol-2 (5 ml, New England Nuclear)–water mixture that formed a stable gel. Duplicate measurements were carried out. The IC50 (concentration which produced 50% inhibition of the enzyme activity) was calculated from regression curves.

*Measurement of inotropic action.* Right ventricular papillary muscles of less than 1 mm in diameter were excised from the heart of male guinea-pigs, 250–320 g, killed by a sharp blow on the skull. The preparations were mounted vertically in an organ bath containing 20 ml of Tyrode solution continuously bubbled with 95% $O_2$ and 5% $CO_2$. One end of each preparation was secured at the bottom of the organ bath and the other was attached to a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co.). The resting length of the muscle was adjusted by applying a stretching force of 500 mg. Isometric change in tension was recorded on a pen-writing oscillograph (Wi-681G, Nihon Kohden Kogyo Co.). Electrical stimulation was applied via the tips of two insulated platinum wires located on the non-tendinous end of the muscle. The stimulus was 2 ms in duration and 1·5× threshold voltage at a frequency of 0·5 Hz. An equilibration period of at least 60 min preceded each experiment and during this the bathing solution was replaced every 20 min with fresh solution. The bath was composed of Tyrode solution of the following composition (mM): NaCl 140; KCl 3; $MgCl_2$ 1; $CaCl_2$ 1·8; $NaH_2PO_4$ 0·4; $NaHCO_3$ 12 and glucose 5 (pH 7·4). Inotropic action was determined by an application of a single concentration of the test compound to one preparation for 60 min. Results are given as percentage of the normal level without any treatment at 60 min after application of the test compound. The EC200 (concentration which produced the 200%-increase in contractility) was obtained from regression curves.

*Chemicals.* γ-Labelled [$^{32}$P]ATP (adenosine 5′-[γ-$^{32}$P] triphosphate triethylammonium salt, specific activity: 3·1 Ci $mmol^{-1}$, Amersham) was further diluted with water. $(Na^+ + K^+)$ATPase (adenosine 5′-triphosphatase: EC 3.6.1.3 from canine kidney, $Na^+$ and $K^+$ activated, ouabain-sensitive, Grade IV, Sigma) was dissolved as described above. Adenosine 5′-triphosphate disodium salt (ATP from equine muscle, Sigma) was dissolved in Milli Q water at a concentration of 30 mM and followed by deionization through Dowex (50W-X4) column. Bufotalin, gamabufotalin, arenobufagin, cinobufagin and marinobufagin were generous gifts from Prof. T. Nambara, Faculty of Pharmaceutical Science, Tohoku University. Ouabain was from Sigma. Compounds to be tested were dissolved in 10% ethanol which was present in a final concentration of 1% or less and this concentration had no effect on parameters studied.

*Results and discussion*

The results are summarized in Table 1. The IC50 (concentration which produced 50% inhibition of $(Na^+ + K^+)$ATPase) for ouabain and gamabufotalin used as reference compounds was calculated to be 2·38 × $10^{-6}$ M and 0·28 × $10^{-6}$ M, respectively. In this respect, gamabufotalin was approximately 10 times more potent than ouabain. As to the novel steroids, the activity of compounds III and XIII was nearly equipotent to ouabain. Compounds I, II, IV, XII and XIV produced relatively low activity among the compounds tested.

All compounds exhibited more or less positive inotropic action in the guinea-pig papillary muscle preparations. Representative tracing (a) and time course (b) of the inotropic action of compound III are illustrated in Fig. 2. All compounds produced a time-dependent inotropic action as did compound III. When the EC200 (concentration which produced 200% increase in the contractility) was compared, gamabufotalin was the most potent. Compound IV produced nearly equipotent positive inotropic action to ouabain.

Table 1. Comparison of $(Na^+ + K^+)$ATPase inhibitory activity and positive inotropic action.

| Test cmpds | $(Na^+ + K^+)$-ATPase ($IC50 \times 10^{-6}$ M) | Positive inotropic action (%) $3 \times 10^{-8}$ M | $10^{-7}$ M | $3 \times 10^{-7}$ M | $10^{-6}$ M | EC200 ($\times 10^{-6}$ M) |
|---|---|---|---|---|---|---|
| Ouabain | 2·38 | 129·2 ± 5·1 (5) | 149·6 ± 3·5 (5) | 195·6 ± 5·4 (5) | 296· ± 20·9 (3) | 0·40 |
| Gamabufotalin | 0·28 | 148·0 ± 8·9 (5) | 185·0 ± 21·0 (5) | 216·3 ± 12·8 (5) | | 0·22 |
| Compound I | 7·27 | | | | 131·0 ± 7·1 (4) | 1< |
| Compound II | 4·57 | | | | 125·0 ± 14·7 (4) | 1< |
| Compound III | 3·13 | | 112·3 ± 3·1 (5) | 142·2 ± 3·4 (5) | 197·0 ± 11·8 (5) | 1·02 |
| Compound IV | 10·1 | | 121·1 ± 5·3 (4) | 159·0 ± 9·9 (4) | 318·2 ± 14·4 (5) | 0·47 |
| Compound XII | 22·0 | | | | 121·5 ± 2·8 (4) | 1< |
| Compound XIII | 1·05 | | 115·4 ± 7·7 (3) | 142·3 ± 15·1 (3) | 216·7 ± 60·6 (3) | 0·84 |
| Compound XIV | 10·4 | | | | 108·5 ± 5·1 (4) | 1< |

Results for positive inotropic action are given as mean ± s.e. 60 min after application of the test solution. Figures in parentheses indicate the number of preparations tested.

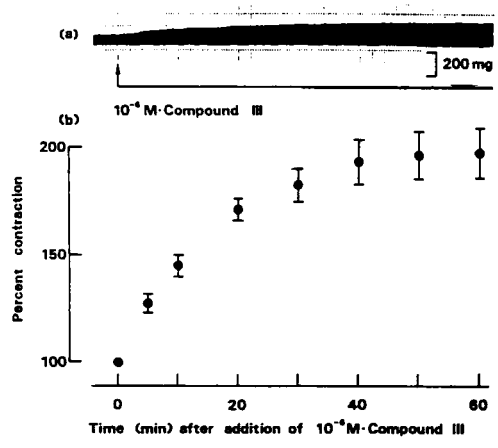


FIG. 2. Representative tracing (a) and time course (b) of positive inotropic action of compound III in guinea pig papillary muscle. Each point represents the mean of 5 observations. Vertical bars show s.e.

The third class of the activity was observed on compounds III and XIII. Compounds I, II, XII and XIV were less active in producing positive inotropic action.

Considering the relationships between the IC50 for $(Na^+ + K^+)$ATPase and the EC200 for positive inotropic action, these values were nearly the same in gamabufotalin and compound XIII. In contrast, IC50 values of ouabain and compounds III and IV were clearly greater than EC200 values of these compounds. Thus, it appears likely that the degree of $(Na^+ + K^+)$ATPase inhibition does not always correlate to the magnitude of the positive inotropic action of these cardiotonic steroids. This speculation may be supported in part by the findings that the correlation coefficient between the enzyme inhibition (%) and the positive inotropic action (%) at $10^{-6}$ M (except for $3 \times 10^{-7}$ M gamabufotalin) was less than 0·6005 in all compounds tested in the present experiments. However, since we used canine kidney $(Na^+ + K^+)$ATPase in the present experiment, this may explain part of the dissociation. According to Ku et al (1974), the degree of $(Na^+ + K^+)$ATPase inhibition is quantitatively related to the magnitude of the inotropic response during the development and dissipation of this effect produced by cardiac glycosides. On the other hand, there are reports (Okita et al 1973; Murthy et al 1974; Peters et al 1974) demonstrating that the inotropic effect is dissociated from the inhibition of $(Na^+ + K^+)$ATPase. Accordingly, it seems that whether the positive inotropic response is associated with the enzyme inhibition remains unsolved. Since we have found that the nuchal glands of *Rhabdophis tigrinus* contain many other compounds (at least 14) which are probably novel steroids differing structurally from known bufodienolides, elucidation of the structure-activity relationships among these novel compounds will help in solving the problems.

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