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26-[^{125}I]Iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors

(steroid hormone/26-iodoponasterone A/*Drosophila*/Kc cells)

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ABSTRACT **The effects of ecdysone, the steroid molting hormone of arthropods, are of considerable interest both to insect physiologists and to those studying steroid-regulated gene expression. Yet progress in understanding ecdysone receptors has been inhibited by the lack of a suitable highly radioactive hormone analog with high affinity for the receptor. Here we report that the synthetic ecdysteroid 26-iodoponasterone A is one of the most active ecdysones known, inducing half-maximal morphological transformation in *Drosophila* Kc167 cells when present at 0.5 nM. 26-[^{125}I]Iodoponasterone A can be prepared at a specific activity of 2175 Ci/mmol (1 Ci = 37 GBq) by reaction of the precursor 26-mesylinokosterone with carrier-free Na^{125}I. The radiolabeled material binds to Kc167 cell ecdysone receptors specifically and with affinity (K_d *ca.* 3.8 × 10^{-10} M). Thus, 26-[^{125}I]iodoponasterone A appears to be a superior radioligand for ecdysone receptors on grounds both of affinity and of specific activity. Its ready availability should greatly facilitate studies of these receptors.**

Steroid hormone receptors are generally detected by the use of high-affinity radioligands. Because such receptors are present at very low concentrations (typically 1–10 ppm of cellular protein), a useful ligand must exhibit not only high affinity for the receptor but also unusually high radiological specific activity. Indeed, the development and use of such radioligands has played an important role in the advancement of our knowledge of the vertebrate steroid receptors (1–7).

Ecdysone,¶ the steroid molting hormone of arthropods, regulates the timing of development and metamorphosis at least in part by its effects on gene expression, effects that are under study in numerous laboratories (9–29). Nevertheless, our knowledge of the molecular biology and physiology of ecdysone receptors is still fragmentary. Ecdysone-binding proteins thought to be ecdysone receptors have been identified in ecdysone-responsive *Drosophila* tissues and cells in culture. These receptors are present at low abundance (*ca.* 1–2 × 10^3 molecules per cell); they exhibit high-affinity binding to active ecdysteroids, the relative affinities being approximately in proportion to relative ecdysone activities (9, 30–38); and their concentration and/or activity is depressed in ecdysone-resistant tissue culture cells (K. Wing, personal communication). Similar receptors have been identified from hypodermal cells of the crayfish *Orconectes* (39).

Because the natural hormone, 20-hydroxyecdysone, is effective only at relatively high concentration (*ca.* 10 nM) and its receptor complex is kinetically unstable, success in studies of the receptor(s) has relied upon use of a more potent analog, the phytoecdysone ponasterone A (40, 41), which is 10–50 times as active in biological assays and which is comparably more active as a ligand (30–38, 42). Thus, the introduction of [^{3}H]ponasterone A (>100 Ci/mmol; 1 Ci = 37 GBq) has been critical to recent progress in the study of ecdysone receptors (31, 36, 38). Still, this material has not been widely available and, given the small amounts of biological material that often must be assayed, is still marginal in its specific activity. Here we report that the synthetic analog 26-iodoponasterone A (called hereafter iodoponasterone) is still more active biologically than ponasterone A, that it can be synthesized readily with carrier-free ^{125}I, and that the radiolabeled material (2175 Ci/mmol) functions well as a ligand for the detection of ecdysone receptors.

MATERIALS AND METHODS

Cells. Kc167 cells were a gift from M. Bourouis (43). We have adapted them to growth in M3 medium (44) supplemented with 5% fetal calf serum.

Bioassay. Ecdysones were bioassayed by their activity in inducing morphological transformation of Kc cells (42). The assay procedure was that described in ref. 42 save that Kc167 cells were used. Ecdysteroids to be tested were dissolved at known concentrations in ethanol [assuming ε_{242} (ethanol) = 12,400 (45)], serially diluted, and tested in 1-ml cell cultures. 20-Hydroxyecdysone, 26-mesylinokosterone, and iodoponasterone were checked for purity by HPLC (see below) and assayed in parallel at concentrations ranging from 10 μM to 10 pM. Bioassays of ^{125}I-substituted steroid were carried out similarly. Dilutions of [^{125}I]iodoponasterone were prepared (assuming a counting efficiency of 50% for ^{125}I in solution), and cells were treated in parallel with it and with a series of concentrations of 20-hydroxyecdysone. The cultures were compared qualitatively under the microscope, the iodoponasterone cultures being assigned positions in the series, and then the 20-hydroxyecdysone series was scored in the usual way.

Steroids. 20-Hydroxyecdysone and muristerone were purchased from Simes (Milan). Ponasterone A was the purified natural product (40). Inokosterone was a gift from T. Takemoto (Tohoku University, Sendai, Japan). All were homogeneous by HPLC save the ponasterone A, which was estimated to be *ca.* 85% pure. 26-Mesylinokosterone and iodoponasterone were synthesized from inokosterone. Details of the syntheses and physical data for these compounds will be reported elsewhere (S.-S.L., K.N., and P.C., unpublished data; inquiries concerning the availability of both compounds should be directed to S.-S.L.).

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¶Ecdysteroid is the generic name for compounds structurally related to α-ecdysone (8). We use ecdysone as the generic name for compounds with the appropriate biological activity in accordance with common usage in the literature and in analogy with the terms estrogen or progestin (9).

[^{125}I]Iodoponasterone Synthesis. Radiolabeled iodoponasterone was prepared from the mesyl intermediate as follows. The label was purchased as carrier-free Na^{125}I (2175 Ci/mmol in dilute base free of reducing agents; Amersham) in lots of 1 or 2 mCi. It was transferred from the shipping vial to a 100-μl conical glass reaction vial by using dry acetone, which subsequently was evaporated under a stream of dry nitrogen gas. (To prepare dry solvents, reagent grade acetone and acetonitrile in lots of a few milliliters were percolated through 2-ml columns of silica gel immediately before use.) 26-Mesylinokosterone (19 nmol/mCi of ^{125}I) was added, and residual water was partially eliminated by repeatedly dissolving the mixture in a small volume of dry acetonitrile and evaporating. Finally the mixture was dissolved in dry acetone (20 μl/mCi of ^{125}I) as reaction solvent, and the reaction vessel was capped tightly and heated to 80°C for 8–12 hr. At the conclusion the acetone was evaporated, and the reaction mixture was dissolved in *ca.* 10 μl of methanol and purified by HPLC. The major radioactive product was eluted at *ca.* 7 ml and cochromatographed with authentic iodoponasterone both by HPLC and by thin-layer chromatography on silica gel. Typical yields have been 12–15%, based on input ^{125}I (six syntheses). Three lots of [^{125}I]iodoponasterone were used in the experiments described here. Each was checked by HPLC and found to be >98% radiochemically pure; residual 26-mesylinokosterone was undetectable by absorbance.

Iodoponasterone solutions are quite stable both radiochemically and chemically when stored over a period of at least 4 months in ethanol at −20°C. We have used successfully and without repurification lots that were 90 days (1.5 half-lives) old. These yielded figures for total binding (at constant input radioactivity) *ca.* 60% of those for freshly labeled iodoponasterone tested in parallel. The details of this decrease in activity have not been investigated.

HPLC. Separations were accomplished with a C_{18} reversed-phase column [Microsorb (Rainin, Woburn, MA) 5 μm, 4.6 mm inside diameter × 25 cm long] equipped with a C_{18} guard column (4.6-mm inside diameter × 1.5 cm). Samples were injected in 2–10 μl of methanol or ethanol and eluted with 70% (vol/vol) methanol/water at 1 ml/min. When appropriate, absorbance was monitored at 254 nm. In this system iodoponasterone was eluted at *ca.* 6.9 ml, whereas 26-mesylinokosterone was eluted at the solvent front (*ca.* 3.6 ml).

Ligand Binding by Intact Cells. Kc167 cells at 1.7 × 10^7 cells per ml were harvested and washed twice by centrifugation in M3 medium without serum. Each incubation tube contained cells (1.75 × 10^8) suspended in 0.5 ml of M3 medium (no serum) containing [^{125}I]iodoponasterone (various concentrations). Each concentration of label was represented by two experimental tubes and two control tubes. The control tubes contained 0.1 mM unlabeled 20-hydroxyecdysone as competitor. Cells were incubated with label for 1 hr and then chilled to 3°C and centrifuged [Microfuge (Beckman)] in the cold. Supernatants were collected for liquid scintillation counting, and the pellets were washed three times with 1 ml of cold saline (46) and then dissolved in Protosol (New England Nuclear) (50 μl) and assayed for radioactivity in 10 ml Aquasol (New England Nuclear). In the presence of unlabeled competitor, the pellets contained 8.8% (SD, 1.0%) of the total input label; this probably corresponds for the most part to internalized label that does not escape during the washes in the cold; the figure approximates that expected given the measured volume of pelleted Kc cells (P.C., unpublished data). For each experimental tube we determined the free ligand by direct measurement of label in an aliquot of the supernatant and considered nonspecifically bound ligand to be 8.8% of the total free ligand. Specific binding was calculated as pellet radioactivity less nonspecific binding.

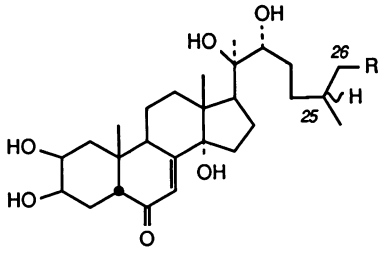
Cell Extracts. Whole-cell extracts have been prepared by procedures slightly modified from those of ref. 36. Data are shown for two extracts similarly prepared. Kc167 cells were harvested at a density of 2 × 10^7 cells per ml, washed extensively in saline, and sonicated in an ice-water bath in 1.25 ml of buffer A [40 mM Hepes, pH 7.0 at 23°C/1 mM dithiothreitol/5% (vol/vol) glycerol]. The sonicate was adjusted to 0.4 M KCl by addition of 2.0 M KCl and incubated for 5 min at 0°C. Finally the extract was centrifuged for 50 min at 20,000 rpm in a Beckman JA 20 rotor. The supernatant was immediately diluted 1:9 with cold buffer A to bring the extract to 40 mM KCl in buffer A. Extract 1 (used in the experiment of Fig. 3) contained 210 μg of protein per 100 μl; extract 2 (experiments of Figs. 4 and 5) contained 380 μg of protein per 100 μl (Bio-Rad protein assay). Extracts were immediately frozen (liquid nitrogen) in 2-ml aliquots and stored at −90°C. Receptor activity remained constant under these conditions for a period of at least 2 months.

Ligand Binding by Extracts. Experimental tubes contained 0.1 volume (typically 14 μl) of [^{125}I]iodoponasterone in buffer A, 0.1 volume of additional buffer A or competitor steroid dissolved in buffer A, and 1 volume of extract. As the competitor steroid, we used muristerone (42)—the most potent ecdysone available to us in quantity—at a final concentration of 4.5 μM. Binding reactions were carried out at room temperature for 1 hr.

Aliquots (10 μl) of each tube were removed and assayed to give a figure for the total concentration of label. Additional aliquots (95–150 μl) were spotted on dry Whatman GF/C filters (2.4 cm) (47). After *ca.* 30 sec, each filter was washed at 3°C in three changes (15 ml each) of 40 mM Hepes, pH 7.0/40 mM KCl. Each wash lasted at least 10–15 min. Finally the wet filters were transferred to scintillation vials and mixed with 1 ml of 4% sodium dodecyl sulfate. Aquasol (10 ml) was added, and radioactivity was determined by liquid scintillation counting.

RESULTS

Iodoponasterone A Is a Potent Ecdysone. Inokosterone (Fig. 1, compound 1) is a phytoecdysone isomeric to 20-hydroxyecdysone and bears a primary hydroxyl group at C-26. The natural product (used here and in all previous work) is a racemic mixture of the two C-25 enantiomers (48–51). By biological assays and by competitive binding using Kc cells, inokosterone has been estimated to be about 10% as active as 20-hydroxyecdysone (33, 42, 52–62). Spindler *et al.* (63) used a bioassay based on puff induction in *Drosophila* salivary glands to investigate the activities of inokosterone C-26 carboxylic acid and its methyl ester. They found that the acid was essentially inactive but that its



1. R = OH
2. R = OMs
3. R = I

FIG. 1. Structures of the ecdysteroids discussed in the text. Compounds: 1, inokosterone; 2, 26-mesylinokosterone; 3, 26-iodoponasterone A.

methyl ester was almost as active as inokosterone itself. This result, along with the activity of other ecdysone analogs like cyasterone [which bears a 5-membered lactone ring at the end of the side chain (42, 64)], suggested the possibility that large nonpolar functional groups at C-26 might be compatible with activity. Thus encouraged, we synthesized iodoponasterone (Fig. 1, compound 3) by way of the intermediate 26-mesylinokosterone (Fig. 1, compound 2) and assayed the biological activities of both compounds by using the Kc cell bioassay (42). 26-Mesylinokosterone proved to be active, though at approximately the same low level as inokosterone itself. In contrast, iodoponasterone was approximately 160-fold more active—i.e., it gave a half-maximal ecdysone response when present at *ca.* 0.5 nM. Thus, at least in the Kc cell bioassay system, iodoponasterone is one of the most potent ecdysones known, exceeded in activity only by 14-desoxymuristerone (65). Since the Kc cell morphological bioassay appears to be an excellent indicator of relative affinities for Kc cell and imaginal disc receptors (compare, for example, refs. 33 and 34), it is reasonable to expect that labeled iodoponasterone should behave as a high-affinity receptor ligand.

[^{125}I]Iodoponasterone A as a Radioligand. Radiolabeled iodoponasterone was synthesized as described. The biological activity of the radioactive material was indistinguishable from that of unlabeled iodoponasterone.

An initial experiment to assess the utility of [^{125}I]iodoponasterone as a ligand is shown in Fig. 2. The figure illustrates saturable binding of iodoponasterone to Kc167 cells. Cells were incubated with increasing concentrations of radioligand at room temperature, then chilled, and washed with cold saline. In such whole-cell assays, there is a substantial component of nonsaturable association, which was assessed from parallel reactions in the presence of 0.1 mM unlabeled 20-hydroxyecdysone. This nonsaturable binding has been subtracted from the data. The figure depicts the saturable component and shows that its maximum is equivalent to *ca.* 4.4×10^4 cpm per 10^7 cells or, assuming a counting efficiency of 50%, about 1100 molecules per cell. Under the same assumption, the effective K_d for cell binding under these conditions is 1.2×10^{-9} M. We conclude that high-affinity binding sites for iodoponasterone are present in Kc167 cells at approximately the abundance expected for ecdysone receptors (9, 30–38).

Next we tested the ability of iodoponasterone to detect ecdysone receptors in cell-free extracts prepared from Kc167 cells. Extracts were prepared by a procedure modified from Sage *et al.* (36). Fresh cells were homogenized and sonicated, first in a low-salt buffer at neutral pH and then in 0.4 M KCl, and the resulting extract was centrifuged and diluted to 40 mM KCl. Protein-bound iodoponasterone was measured by the procedure of Cousens and Eskin (47): reaction mixtures containing complexes are spotted on glass fiber filters that retain proteins efficiently; stable ligand–receptor complexes survive vigorous washing in the cold. We found that this procedure yields very low nonsaturable backgrounds (typically 0.3% of input cpm) with *Drosophila* cell extracts and [^{125}I]iodoponasterone.

Fig. 3 shows the saturable binding of [^{125}I]iodoponasterone by one cell extract. Once again nonsaturable binding has been subtracted. Evidently the extract contains a component or components that bind iodoponasterone with K_d = *ca.* 5.6×10^5 cpm/ml and are saturable. Once again with the assumption of a counting efficiency of 50%, the calculated K_d is equivalent to 2.3×10^{-10} M, and the maximum binding is *ca.* 15 fmol/mg of protein.

High-affinity steroid–receptor complexes should dissociate slowly; as a practical matter, filter-binding assays depend upon this property. Fig. 4 confirms that at 0°C, bound [^{125}I]iodoponasterone exchanges only slowly (half-time *ca.* 20 hr) with unlabeled muristerone present in vast (33,000-fold) molar excess. The figure also shows that under these conditions there is negligible loss of receptor activity over 2 days.

Finally, we have tested the specificity of [^{125}I]iodoponasterone binding by competition with unlabeled iodoponasterone and other ecdysones. The results (Fig. 5) are independent of assumptions concerning counting efficiency and indicate a K_d for iodoponasterone of *ca.* 3.8×10^{-10} M, 1/2 to 1/3rd that for ponasterone A and *ca.* 1/100th that for 20-hydroxyecdysone. These relative binding activities reflect faithfully the relative biological activities described

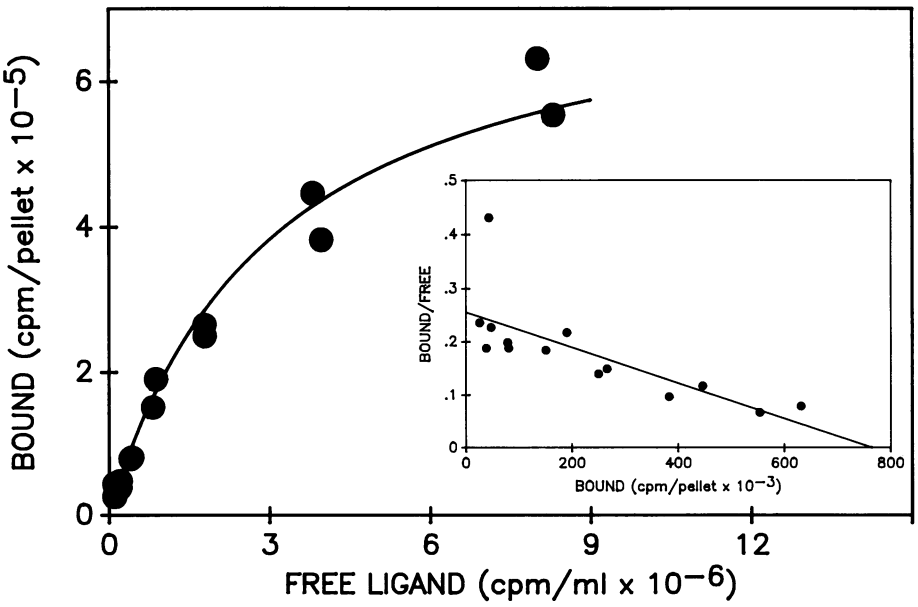


FIG. 2. Binding of [^{125}I]iodoponasterone to intact Kc167 cells. The ordinate represents saturable binding—i.e., the portion of total binding blocked by excess unlabeled 20-hydroxyecdysone. Each point in the figure represents a single experimental tube. (*Inset*) Scatchard plot of the data and the calculated first-order regression line; the inferred parameters are: maximum binding = 4.4×10^4 cpm per 10^7 cells and $K_d = 3.0 \times 10^6$ cpm/ml. These correspond approximately (see text) to a K_d of 1.2×10^{-9} M and maximum binding of *ca.* 1100 molecules per cell. The curve in the main figure is the theoretical curve for noncooperative binding given these parameters.

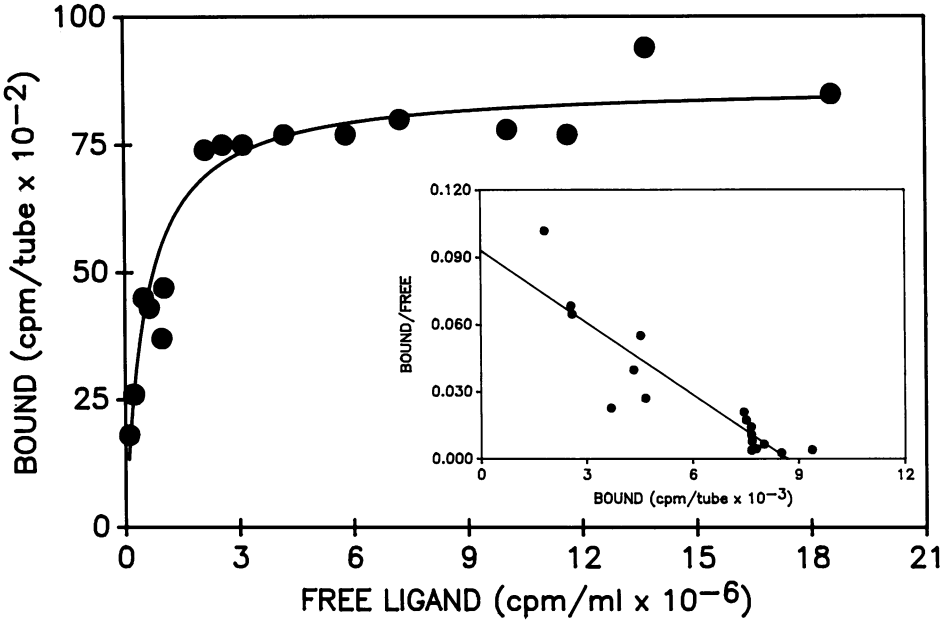


FIG. 3. Binding of [^{125}I]iodoponasterone by a cell extract. Once again, the ordinate represents saturable binding. Reactions were carried out in a total volume of 168 μl; control tubes contained muristerone (42) at 4.5 μM as competitor. Binding reactions were 1 hr at room temperature, and 150-μl aliquots were spotted on the filters. Control filters retained 0.29% (SD, 0.07%) of the input cpm. For each experimental tube, free ligand was determined as the difference between total cpm and bound cpm; the plotted figures represent total bound cpm less the nonspecific component. Each point in the figure represents a single experimental tube. (*Inset*) Scatchard plot and its regression line; the resulting binding parameters are: K_d = 5.6 × 10^5 cpm/ml and maximum binding = 8680 cpm per tube, corresponding to ≈2.3 × 10^{-10} M and *ca.* 15 fmol/mg of protein. The curve in the main figure corresponds to theoretical binding given these parameters.

above. Thus, the results are fully compatible with the idea that the binding component(s) detected by [^{125}I]iodoponasterone are ecdysone receptors.

DISCUSSION

Our results suggest that [^{125}I]iodoponasterone should prove useful as a specific radioligand for detection of ecdysone receptors. Whereas iodoponasterone binds receptors only marginally better than does ponasterone A, it offers the substantial advantage that it can be prepared inexpensively and conveniently at 15- to 20-fold higher specific activity. Arrangements are being made for the ready availability of the stable intermediate 26-mesylinokosterone by S.-S.L. The specific activity of the radioligand can be taken as constant at 2175 Ci/mmol, the decay products are nonradioactive, and, at the concentrations present in samples up to

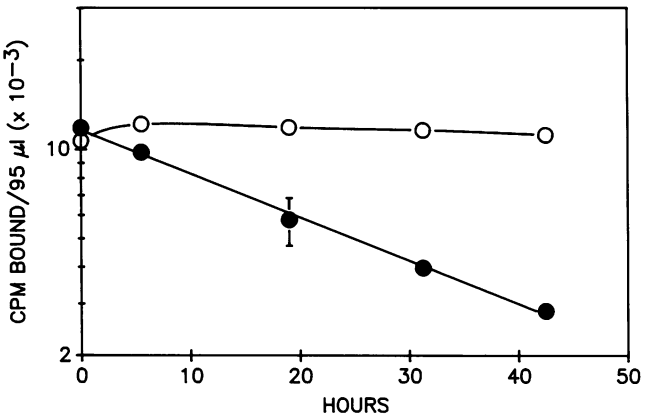


FIG. 4. Kinetics of exchange at 0°C. Standard binding reactions were carried out at 6.03 × 10^5 cpm/ml for 1 hr. Specific binding by 95-μl aliquots averaged 11,850 cpm, and the background unaffected by competition averaged 110 cpm. Tubes A and B were experimental tubes (1.65 ml each) incubated in parallel and then chilled to 0°C. Each reaction was diluted with 150 μl of ice-cold buffer A; then tube B was made 8.3 μM in unlabeled muristerone. The tubes were maintained at 0°C, and standard filter binding assays (95-μl aliquots) were performed at intervals. Tube A (○) monitors the stability of the receptor(s) under these conditions, while tube B (●) monitors the exchange of unlabeled for labeled steroid. Each datum represents two measurements; where the range exceeds the size of the symbols, it is indicated.

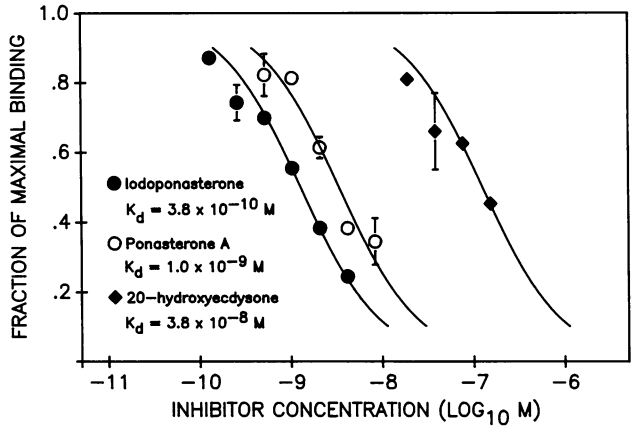


FIG. 5. Competitive inhibition of [^{125}I]iodoponasterone binding by active ecdysones. The ordinate represents the fraction of maximal specific binding, where maximal specific binding is taken as the cpm bound in the absence of competitor (18,000; n = 6, SD = 300) less the cpm bound in the presence of 4.5 μM muristerone as competitor (760; n = 6, SD = 100). Each datum was determined in duplicate; where the range of the two observations exceeds the size of the symbols, it is indicated. Reactions (120 μl) included 1.57 × 10^5 cpm of [^{125}I]iodoponasterone and various concentrations of unlabeled ecdysteroids. After a 1-hr incubation at room temperature, 95-μl aliquots were spotted on filters and washed. Raw data were reduced to a series of logit plots to define the relative K_ds: iodoponasterone, 1; ponasterone A, 2.6; 20-hydroxyecdysone, 98. Absolute K_ds follow from the free ligand concentration, 1.31 × 10^6 cpm/ml, equal to 2.36 times K_d (Fig. 3). The curves on the graph represent theoretical inhibition curves given the parameters.

1–1.5 half-lives old, they appear to have only a slight effect on binding reactions. It is also potentially a convenience that [^{125}I]iodoponasterone can be detected by γ counting and autoradiography.

We expect that the sensitivity of this radioligand will facilitate studies of ecdysone receptors and their molecular actions. In addition, we note that, because of that sensitivity, it may become feasible for insect physiologists and molecular biologists to measure ecdysone receptor levels in individual organs of single small insects.

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