High specific activity enantiomerically enriched juvenile hormones: Synthesis and binding assay

(asymmetric epoxidation/juvenile hormone binding protein/juvenile hormone I/tritium labeling/*Manduca sexta*)

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ABSTRACT   A stereoselective total synthesis of chiral juvenile hormone I is described that allows stoichiometric introduction of two tritium atoms in the final step. Both optical antipodes of the pivotal epoxy alcohol intermediate were prepared in 95% enantiomeric excess by the Sharpless epoxidation of a (Z)-allylic alcohol. Elaboration of the hydroxymethyl group to a vinyl group followed by selective homogeneous tritiation affords optically active juvenile hormone I analogs at 58 Ci/mmol. Competitive binding of the labeled 10R,11S and 10S,11R enantiomers with unlabeled enantiomers to the hemolymph binding protein of *Manduca sexta* larvae was determined by using a dextran-coated charcoal assay. The natural 10R,11S enantiomer has twice the relative binding affinity of the 10S,11R enantiomer. The availability of such high specific activity optically pure hormones will contribute substantially to the search for high-affinity receptors for juvenile hormones in the nuclei of cells. Moreover, the chiral 12-hydroxy-(10R,11S)-epoxy intermediate allows modification of juvenile hormone for solid-phase biochemical and radioimmunochemical work without altering either the biologically important carbomethoxy or epoxy recognition sites.

Molecular studies of the endocrinology of juvenile hormone action in insects is limited by the availability of synthetic hormones and hormone analogs of sufficient optical purity and specific activity (1, 2). Currently, the commercially available juvenile hormones are all racemic, and the 10-³H-labeled racemates have maximum specific activities of 11–15 Ci/mmol (1 Ci = 37 GBq). These materials are inadequate for the identification of putative low-abundance high-affinity juvenile hormone receptors with dissociation constants <1 nM, and the presence of the unnatural enantiomer could possibly lead to competitive displacement of the natural analog or to excessively high nonspecific binding in the assays used (3).

Optically pure (10R,11S)-juvenile hormone I (JH-I), specific activity 53 Ci/mmol, has been produced enzymatically by using the O-methyltransferase of male *Hyalophora cecropia* accessory glands to esterify enantioselectively the 10R,11S antipode of racemic JH-I acid with S-[methyl-³H]adenosylmethionine (4). The JH-I acid is obtained from the action of a crude juvenile hormone esterase preparation on racemic JH-I. This procedure has several drawbacks: (*i*) it has not made optically pure high specific activity JH-I routinely available to most investigators, (*ii*) the labile methoxyl-³H can cause serious background problems, and (*iii*) the label is not suitably placed for chemical modification into a tritium-labeled photoactivatable analog of JH-I homologous to epoxyfarnesyl diazoacetate (5, 6). Natural (10R,11S)-JH-I (7, 8) has been totally synthesized by two groups (9, 10) using optically active ketals, but neither of these routes allows the

facile introduction of tritium at high specific activity in the final or penultimate steps. JH-III has been prepared in chiral form by resolution of the (+)-α-methoxy-(α)-trifluoromethylphenylacetate (MTPA) (11) or 3β-acetoxyetienate (12) esters, or with a fungal extract (ref. 12 and references therein), but these methods are also impractical for obtaining synthetic chiral labeled hormones.

We now report the total synthesis of >95% enantiomerically enriched (10R,11S)-JH-I, its 10S,11R antipode, and the corresponding 12,13-³H-labeled isotopomers with a specific activity of 58 Ci/mmol. The synthesis is versatile in providing access to both antipodes of JH-I and JH-II, and in furnishing a new functionality for use in tethering juvenile hormone to proteins and to solid supports. Furthermore, we report the confirmation of the authenticity and utility of these radiolabeled and radioinert JH-I enantiomers in competitive binding assays with the larval hemolymph juvenile hormone binding protein of *Manduca sexta* (13).

MATERIALS AND METHODS

Synthesis. The synthetic procedures and key spectral and physical properties for isolated compounds in the later stages of the sequence are summarized below and in Fig. 1. Full experimental details will be presented elsewhere.

(E,E,Z)-1-t-Butyldimethylsiloxyl-12-tetrahydropyranyloxy-3,11-dimethyl-7-ethyl-2,6,10-dodecatriene (compound 4). Silyl ether (compound 3; 0.57 mmol) was kept for 24 hr (0°C) with 1.5 equiv. of p-toluenesulfonyl chloride in pyridine, and the crude tosylate was extracted with ethyl acetate/hexane (1:4), washed (H₂O, saturated CuSO₄), chromatographed, and then stirred for 12 hr at 20°C with excess sodium iodide in acetone. The chromatographed iodide was then refluxed in acetonitrile with 1.1 equiv. of triphenylphosphine, and the crude phosphonium salt was triturated with hexane. The ylid was formed by addition of 1.1 equiv. of n-butyllithium to a tetrahydrofuran solution of the phosphonium salt at −25°C to 0°C, cooled to −70°C, and then tetrahydropyranyloxyacetone (2.0 equiv.) in tetrahydrofuran was added (14, 15). After warming to 20°C (1 hr), the usual workup afforded triene ether (compound 4) in 65% overall yield from compound 3 as a 97:3 mixture of Z/E stereoisomers (GC, Durabond DB-5, 30 m × 0.25 mm, 240°C). The first eluting Z isomer could be obtained in >98% geometric purity by flash chromatography (16): ¹H-NMR (CDCl₃), δ [ppm from (CH₃)₄Si] 0.06 (s, SiMe₂), 0.90 (s, tBu), 0.95 (t, J = 7.5 Hz, 7-CH₂CH₃), 1.62 (m, 3-CH₃), 1.75 (m, 11-CH₃ of Z isomer), 4.09 (m, H-12 of Z isomer), 4.18 (d, J = 5.7 Hz, H-1), 4.59 (m, THP ketal CH), 5.08 (t, J = 7 Hz, H-6), 5.2–5.4 (br m, H-2, 10).

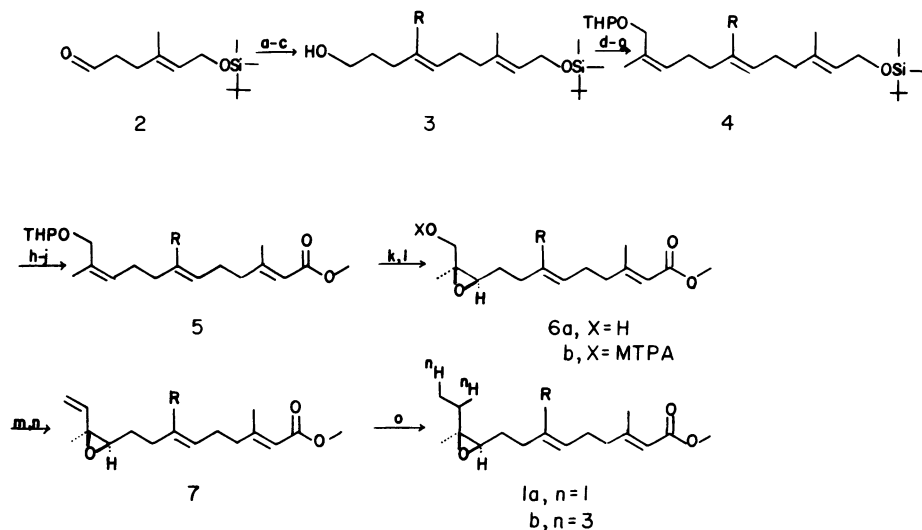Biochemistry: Prestwich and Wawrzeńczyk

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FIG. 1.   Asymmetric synthesis of ^{3}H-labeled JH-I enantiomers (R = C_2H_5). Reagents: a, 2-(1-butenyl)magnesium bromide, tetrahydrofuran, $-78°C$; b, $(C_2H_5O)_3CCH_3$, $CH_3CH_2CO_2H$ (cat.), $130°C$, 4 hr; c, LiAlH$_4$, tetrahydrofuran; d, p-toluenesulfonyl chloride, pyridine, $0°C$, 24 hr; e, NaI, acetone, $25°C$; f, Ph$_3$P, CH$_3$CN, reflux; g, n-BuLi, tetrahydrofuran, tetrahydropyranyloxyacetone; h, NBu$_4$$^+F^-$, tetrahydrofuran; i, MnO$_2$, hexane; j, CH$_3$CO$_2$H, CH$_3$OH, NaCN, MnO$_2$; k, pyridinium p-toluenesulfonate, CH$_3$OH; l, Ti(OiPr)$_4$, t-BuOOH, CH$_2$Cl$_2$; (+)-diethyl tartrate; m, CrO$_3$·2Py, CH$_2$Cl$_2$; n, Ph$_3$P = CH$_2$, tetrahydrofuran; o, H$_2$, Rh(Ph$_3$P)$_3$Cl, benzene; $n = 1,3$.

Methyl (E,E,Z)-12-tetrahydropyranyloxy-3,11-dimethyl-7-ethyl-2,6,10-dodecatrienoate (compound 5). Selective removal of the silyl protecting group from 0.50 mmol of the silyl ether (compound 4) with 1.0 mmol tetra-n-butylammonium fluoride in tetrahydrofuran ($20°C$, 1 hr) gave a trienol, which was chromatographed, oxidized to the trienal with MnO$_2$ (10 mmol), and then converted to the methyl trienoate with MnO$_2$ (10 mmol) in the presence of acetic acid (1.5 mmol), sodium cyanide (5 mmol), and methanol (17) to give, after chromatography, a 46% yield (from compound 4) of ester (compound 5): ^{1}H-NMR(CDCl$_3$), δ 1.54 (s, 3-CH$_3$), δ 3.68 (s, CO$_2$CH$_3$), 5.66 (br s, H-2).

Methyl (E,E)-(10R,11S)-3,11-dimethyl-10,11-epoxy-7-ethyl-12-hydroxy-2,6-dodecadienoate (compound 6a). Removal of the tetrahydropyranyl ether (compound 5) with pyridine p-toluenesulfonate in methanol gave, after chromatography, 95% of the 12-hydroxy compound. Asymmetric epoxidation was carried out under the Katsuki–Sharpless conditions (18), by addition of 0.12 mmol of this allylic alcohol in 2 ml of CH$_2$Cl$_2$ to a solution of 0.16 mmol of (+)-diethyltartrate, 0.25 mmol of 1 M t-butylhydroperoxide in CH$_2$Cl$_2$, and 0.14 mmol of titanium tetraisopropoxide in methylene chloride at $-20°C$. The product was isolated and chromatographed to afford 79% of the epoxy alcohol (compound 6a): ^{1}H-NMR(CDCl$_3$) δ 1.37 (s, 11-CH$_3$), 2.85 (t, $J = 6.4$ Hz, H-10), 5.10 (m, H-6), 5.66 (m, H-2). The (10S,11R)-epoxy alcohol was obtained similarly by using (−)-diethyltartrate as the chiral auxiliary.

Derivatization of a 2-mg sample of each epoxy alcohol (compound 6a) with (+)-methoxytrifluoromethylphenylacetyl chloride (MTPA-Cl) (19) in CH$_2$Cl$_2$ with N,N-dimethyl-4-aminopyridine gave, after chromatography, the diastereomeric MTPA esters (compound 6b), which were analyzed by ^{1}H-NMR to determine enantiomeric purity. Fig. 2 shows the ^{1}H-NMR regions for the C-12 methylene protons of the MTPA esters of the 10R,11S and 10S,11R epoxy alcohols. The methoxy signals appear as poorly resolved ($\Delta\delta = 0.01$ ppm) quartets ($^5J_{HF} = 0.8$ Hz), but the two different AB quartets of the C-12 protons could be integrated separately to obtain the value of the (10R,11S)/(10S,11R) ratio of 98:2 for the 10R,11S sample and 3:97 for the 10S,11R sample.

Methyl (E,E)-(10R,11S)-3,11-dimethyl-10,11-epoxy-7-ethyl-2,6,12-dodecatrienoate (compound 7). The epoxy alcohol was oxidized with *in situ* Collins reagent (chromium

trioxide/pyridine) in methylene chloride (20) to give, after chromatography on silica gel, the epoxy aldehyde: ^{1}H-NMR(CDCl$_3$), δ 1.39 (s, 11-CH$_3$), 3.05 (t, $J = 6.5$ Hz, H-10), 9.35 (s, H-12).

The purified aldehyde was added in tetrahydrofuran to a solution of excess methylenetriphenylphosphorane in tetrahydrofuran at $-78°C$ and stirred for 1 hr ($-78°C$ to $20°C$). Workup and chromatography gave the homogeneous (10R,11S)-epoxytriene ester (compound 7) in 61% from compound 6a. The (10S,11R)-triene ester was obtained similarly: ^{1}H-NMR(CDCl$_3$), δ 1.41 (s, 11-CH$_3$), 2.87 (t, $J = 6.3$ Hz, H-10), 5.1–5.8 (ABC pattern, H-12, 13).

JH-I. A solution of the 12,13-dehydro JH-I (compound 7) (2.0 mg, 7.0 μmol) in 1 ml of benzene was added to 1 ml of benzene containing 7.2 mg (7.7 μmol) of tris(triphenylphosphine)chlororodium (Wilkinson's catalyst) (21) that had been prereduced with hydrogen at atmospheric pressure to give the catalytically active dihydro form. Failure to prereduce the catalyst resulted in no reaction or, in the presence of methanol cosolvent, in apparent rearrangement of the vinyloxirane to an enone. After 2 hr at $20°C$, the benzene was evaporated, the residue was dissolved in 1.5% ethyl acetate/hexane, and the crude JH-I was purified by flash chromatography, eluting with 5% ethyl acetate/hexane, which was spectrally and physically identical with commercial JH-I.

[12,13-^{3}H$_2$]JH-I. A solution of 7.60 mg (8.3 μmol) of tris(triphenylphosphine)chlororodium in 2 ml of benzene was freeze–thaw degassed on the tritium line at the National Tritium Labeling Facility. (A second identical solution was processed simultaneously on a second port for tritiation of the 10S,11R enantiomer.) The line was charged with $\approx$200 Ci of carrier-free tritium gas (desorbed from U^{3}H$_3$) and was maintained at 740 torr during 2 hr of vigorous stirring to prereduce the catalyst. A solution of 1.97 mg (6.9 μmol) of (10R,11S)-dehydro JH-I in 1.0 ml was added to one flask and 2.02 mg (6.9 μmol) of (10S,11R)-dehydro JH-I was added to the other, and stirring under ^{3}H$_2$ was continued for 2 hr at $20°C$. The reactions were frozen (liquid N$_2$), degassed, and concentrated *in vacuo* to 1 ml; hexane (5 ml) was added to precipitate the catalyst, which was then removed by filtration through a 0.4-μm glass-fiber filter. The samples were lyophilized, redissolved in 1 ml of 1.5% ethyl acetate/hexane, and chromatographed with bulb pressure over 230–400 mesh
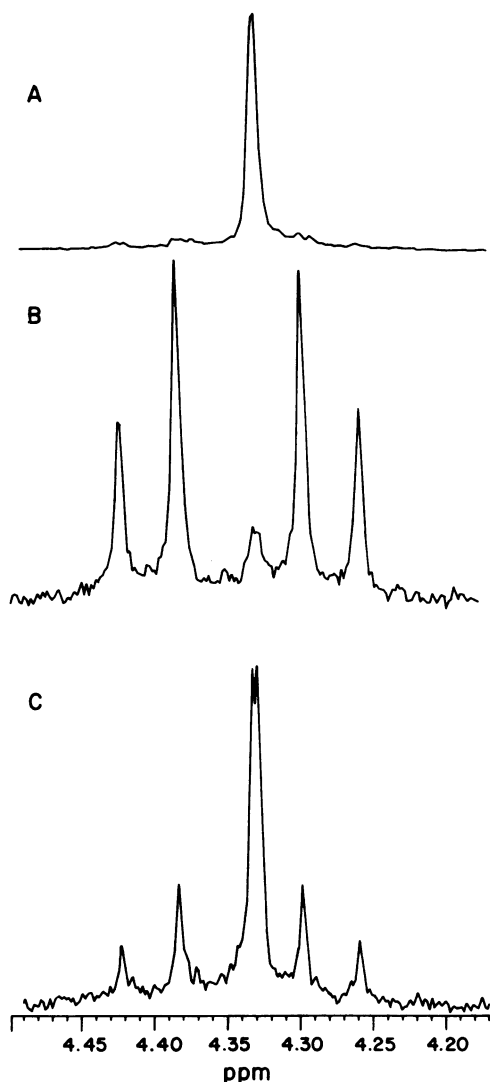
FIG. 2. Determination of enantiomeric purity of epoxy alcohols (compound 6a) by ^{1}H-NMR of the (+)-MTPA esters (compound 6b). The C-12 methylene protons appeared as AB quartets, which could be separately integrated. The 10R,11S (compound 6b) had a >95% enantiomeric excess as seen in A; ^{1}H-NMR (300 MHz), $\Delta\nu = 4.95$ Hz ($\Delta\delta = 0.016$ ppm), $J_{AB} = 11.88$ Hz. The 10S,11R enantiomer (compound 6b) was also >95% enantiomeric excess (B); $\Delta\nu = 35.31$ Hz ($\Delta\delta = 0.12$ ppm), $J_{AB} = 11.68$ Hz. (C) Admixture of 79% 10R,11S and 21% 10S,11R obtained when the epoxidation was conducted at 0°C using (+)-diethyltartrate.

silica gel in a disposable pipette. JH-I was monitored by TLC on MN SilG UV-254 4 × 8 cm plates, eluted with 20% ethyl acetate/hexane, and visualized with UV light and with I_2 (R_f, dehydro JH-I = 0.33; R_f, JH-I = 0.30). Three fractions totaling 318 mCi of natural (10R,11S)-JH-I (80% radiochemical yield at 58 Ci/mmol) and, from the other reaction, three fractions totaling 297 mCi of unnatural (10S,11R)-JH-I (74% yield) were obtained. For each antipode, the first fraction contained a trace of R_f 0.33, which could be either starting material or a rearranged enone.

All six fractions were analyzed by HPLC (22, 23) using a radially compressed 8 × 150 mm column of 10-μm C_{18}-coated silica particles and a convex gradient from 75% CH_3OH/H_2O to 100% CH_3OH. Effluent was monitored continuously at 220 nm and diode-array UV spectra were stored at 0.64-sec intervals during 20-min runs (HP1040A); commercial JH-I from Sigma was used as the standard. An in-line radiochemical monitor (Berthold) was also used, showing that the radiochemical purity of the natural and unnatural JH-I

antipodes exceeded 98% for all fractions. However, a non-radioactive faster-moving shoulder, $\lambda_{max} = 230$ nm, showed ≈30% of the absorption at 220 nm in the first fraction of each enantiomer and <5% of the 220 nm absorption in the next two fractions. The middle fractions were concentrated under N_2, dissolved in benzene/hexane, and aliquots were taken for binding assays.

Binding Assays. *Manduca sexta* were reared from eggs and fourth instars with slipped head capsules were bled through the anal proleg into ice-cooled tubes containing phenyl-thiourea to inhibit tyrosinase action. Hemocytes were removed by centrifugation (30,000 × g; 20 min), the hemolymph was incubated with 5 vol of 0.2 mM phenylmethyl-sulfonyl fluoride in 20 mM Tris·HCl buffer/100 mM NaCl/0.2 mM phenylthiourea, pH 7.4, concentrated to its original volume by ultrafiltration, and then chromatographed on Ultrogel AcA44 to give partially purified binding protein (24). Juvenile hormone binding was determined by modification of the dextran-coated charcoal assay as described by Kramer *et al.* (25) and by Goodman *et al.* (13). All commercial and synthetic juvenile hormones used were repurified by silica gel flash chromatography before use and were stored in hexane. Concentrations were determined from UV absorbance at 217 nm ($\varepsilon = 14,770$ for JH-I, -II, -III) in ethanol. Dilutions were freshly prepared from stock solutions containing <0.5% ethanol in buffer (pH 7.4) (10 mM Tris·HCl/10 mM KCl). Standard precautions, including polyethylene glycol-coating of glassware, were taken for aqueous juvenile hormone solutions (6). The assay protocol is described in the Fig. 3 legend.

RESULTS AND DISCUSSION

The synthesis follows known stereoselective reaction sequences for the elaboration of the trisubstituted olefins and for the asymmetric epoxidation of allylic alcohols (Fig. 1). Thus, controlled ozonolysis (using 1.2 equiv. of O_3 as a saturated solution in CH_2Cl_2 at −78°C) (26) of the *t*-butyldimethylsilyl ether of >98% *trans*-geraniol in CH_3OH-CH_2Cl_2 with $(CH_3)_2S$ workup leads to aldehyde (compound 2) in 30–50% chromatographed yield. Alkylation with 2-(1-butenyl)magnesium bromide followed by Claisen orthoester rearrangement and then hydride reduction leads to the alcohol (compound 3), where R = C_2H_5 (64% from compound 2). The hydroxyl group is transformed in three steps to the crystalline phosphonium salt; the ylid is then generated with butyllithium and condensed with the tetrahydropyranyl ether of hydroxyacetone to give a 97:3 mixture of Z/E triene diethers (compound 4) (65% from compound 3). Removal of the *t*-butyldimethylsilyl group with fluoride and a two-step oxidation of the allylic alcohol to the aldehyde and then directly to the methyl ester was achieved in 46% from compound 4. Removal of the tetrahydropyranyl ether and asymmetric epoxidation with titanium isopropoxide and *t*-butylhydroperoxide in the presence of (+)-diethyltartrate afforded the (10R,11S)-epoxy alcohol (compound 6a) (75% from compound 5). The 10S,11R enantiomer of JH-I was prepared similarly from compound 5 in 88% yield with (−)-diethyl tartrate as the chiral ligand. The enantiomeric excesses were determined by separate integration of the carbinyl methylene resonances (two AB quartets) and the MTPA methoxy quartets of the two diastereoisomeric (+)-MTPA esters (compound 6b). The natural 10R,11S antipode and the unnatural 10S,11R antipode were both obtained in 95% enantiomeric excess. The chiral epoxides were then oxidized (*in situ* Collins method) and methylenated to give the vinyl epoxide (compound 7) (61% from compound 6a). The unlabeled hormones were obtained by selective homogeneous hydrogenation in the presence of Wilkinson's cata-
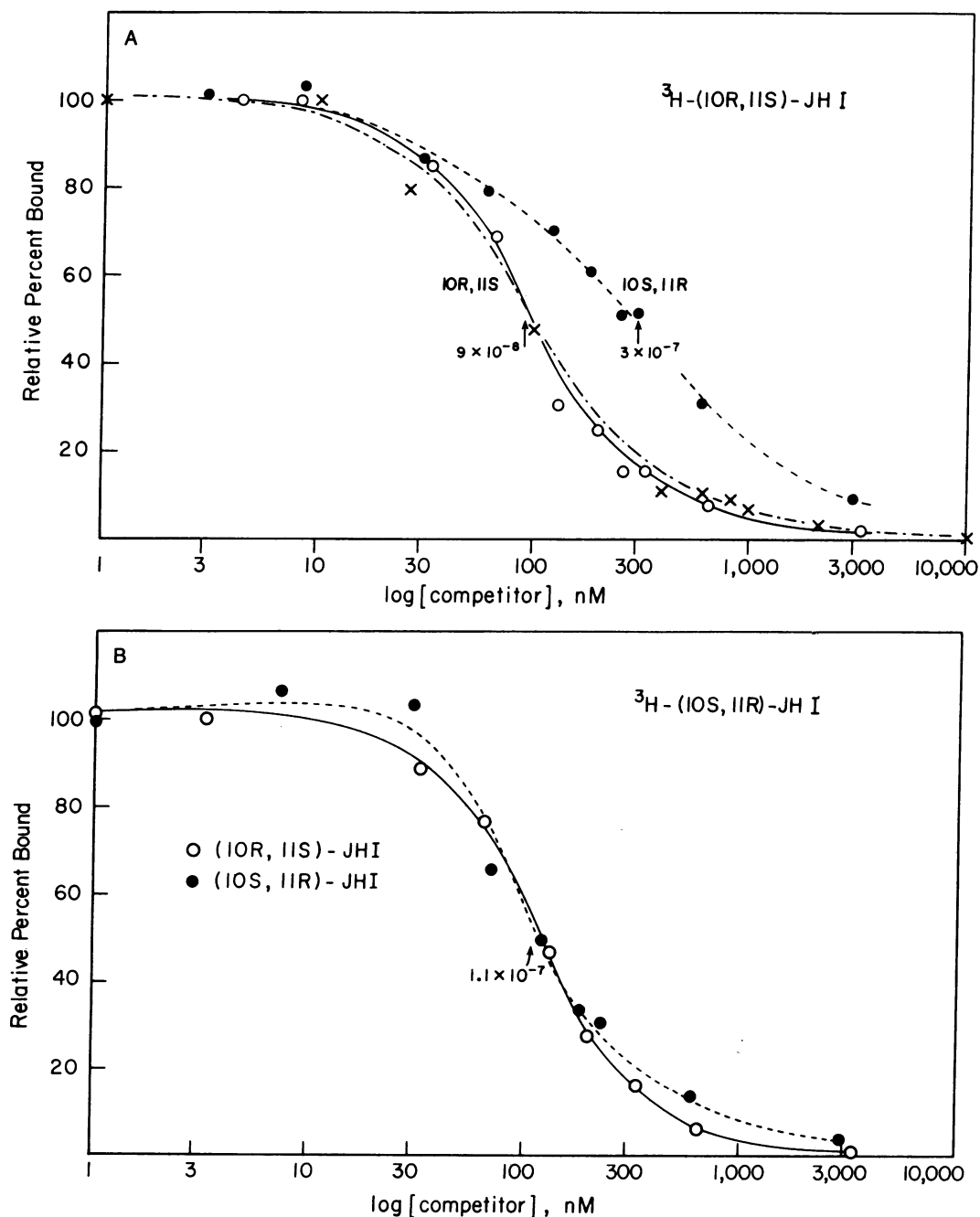
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FIG. 3. Competitive displacement of ^{3}H-labeled JH-I enantiomers (0.8 nM) by unlabeled JH-I enantiomers. The relative binding of the labeled 10R,11S isomer is shown in A as a function of the concentration of unlabeled 10R,11S (○) and (10S,11R)-JH-I (●). ×, Competition by racemic JH-I. (B) Analogous experiment for the ^{3}H-labeled 10S,11R isomer. Buffer (50 μl) or a solution of unlabeled competitor (50 μl) (1 nM to 40 μM) was added to a 6 × 50 mm PEG-coated glass tube, followed by 100 μl of *M. sexta* 4th instar hemolymph juvenile hormone binding protein (diluted 1:4) (from gel filtration) containing 2 μM 3-octylthio-1,1,1-trifluoro-2-propanone) as a juvenile hormone esterase inhibitor (27, 28). After mixing in a Vortex and incubating at 4°C for 2 hr, 50 μl of 3.2 nM [^{3}H]JH-I in 10 mM Tris·HCl/10 mM KCl, pH 7.4, was added and the tubes were mixed in a Vortex and incubated 4–8 hr at 4°C. Then, 100 μl of 1.1% dextran-coated charcoal was added, tubes were mixed in a Vortex, incubated 15 min at 4°C, and centrifuged (12,000 × g, 5 min), and two 100-μl aliquots were withdrawn for scintillation counting. "No protein" blanks were subtracted and a "no charcoal" sample gave total counts present. Protein concentration was selected to give 50% binding of 0.8 nM ^{3}H-labeled (10R,11S)-JH-I (final concentration) after 4–8 hr equilibration.

lyst, Rh(Ph$_3$P)$_3$Cl. Unlabeled optically active JH-I enantiomers obtained in this fashion were used in the competitive binding experiments described below.

The binding assays confirm the identities of the new synthetic JH-I enantiomers and are consistent with the original determination (8) of the 10R,11S enantiomer as the natural JH-I stereoisomer. Use of the partially purified protein eliminated virtually all nonspecific binding. At 4°C and 4 hr of incubation with 0.8 nM [^{3}H]JH-I isomers, 57.0%

of the 10R,11S enantiomer was bound while only 29.8% of the 10S,11R isomer was bound. These percentage bound values are in the same ratio as the relative binding affinities estimated from the competition curves in Fig. 3. These apparent K_d values are consistent with values obtained by Kramer *et al.* (25) and Goodman *et al.* (13) for racemic materials. This [^{3}H]JH-I concentration gives a total of ≈20,000 dpm per 100-μl aliquot of assay. Moreover, the actual concentration of ^{3}H-labeled (10R,11S)-JH-I in the Kramer and Goodman

assays is 2–4 nM, and it is contaminated with labeled (10*S*,11*R*)-JH-I and both unlabeled stereoisomers.

In contrast to the JH-III enantiomers, which differ in relative binding affinity by nearly a factor of 100, the more lipophilic JH-I enantiomers differ by only a factor of 2. Apparently the sensitivity to lipophilicity overrides the sensitivity to epoxide absolute configuration for this protein. Curiously, the competitive abilities of the two enantiomers are essentially equivalent when unnatural 12,13-^{3}H$_2$-labeled (10*S*,11*R*)-JH-I is used as the ligand.

SUMMARY AND PROSPECTUS

Optically active JH-I of high specific activity (58 Ci/mmol) is now available in a commercially exploitable form. This method has also been extended to include optically active JH-II (Fig. 1, R = CH$_3$) by substitution of the Grignard reagent from 2-bromopropene for that from 2-bromo-1-butene (unpublished results) in the conversion of compound 2 to compound 3. The key substances for covalent modification of juvenile hormone receptors are the photoaffinity labels homologous to epoxyfarnesyl diazoacetate (unpublished results). The synthetic details of these procedures will be published elsewhere. We hope that insect endocrinologists will adopt these new materials to further understand the molecular basis of juvenile hormone action in insects.

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