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Binding of *de novo* synthesized radiolabeled juvenile hormone (JH III) by JH receptors from the Cuban subterranean termite *Prorhinotermes simplex* and the German cockroach *Blattella germanica*

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ABSTRACT

Juvenile hormone (JH) controls insect reproduction and development through an intracellular receptor complex comprising two bHLH-PAS proteins, the JH-binding Methoprene-tolerant (Met) and its partner Taiman (Tai). Many hemimetabolous insects including cockroaches strictly depend on JH for stimulation of vitellogenesis. In termites, the eusocial hemimetabolans, JH also regulates the development of caste polyphenism. Studies addressing the agonist ligand binding to recombinant JH receptors currently include three species belonging to two holometabolous insect orders, but none that would represent any of the hemimetabolous orders. Here, we examined JH receptors in two representatives of Blattodea, the cockroach *Blattella germanica* and the termite *Prorhinotermes simplex*. To test the JH-binding capacity of Met proteins from these species, we performed chemical synthesis and tritium labeling of the natural blattodean JH homolog, JH III. Our improved protocol increased the yield and specific activity of [10-^{3}H]JH III relative to formerly available preparations. Met proteins from both species specifically bound [^{3}H]JH III with high affinity, whereas Met variants mutated at a critical position within the ligand-binding domain were incapable of such binding. Furthermore, JH III and the synthetic JH mimic fenoxycarb stimulated dimerization between Met and Tai components of the respective JH receptors of both species. These data present primary evidence for agonist binding by JH receptors in any hemimetabolous species and provide a molecular basis for JH action in cockroaches and termites.

1. Introduction

Hemimetabolous insects have been instrumental for research on juvenile hormone (JH) signaling in insect reproduction and development (Wyatt and Davey, 1996; Bellés et al., 2005; Jindra et al., 2013; Santos et al., 2019; Riddiford, 2020; Martín et al., 2021; Wu et al., 2021). Following early discoveries in the hemipteran *Rhodnius prolixus* (Wigglesworth, 1934, 1936, 1940) on the roles of JH and its source, the *corpora allata* glands, important insights have been obtained using cockroaches (Blattodea). Cockroaches such as *Diploptera punctata*, *Nauphoeta cinerea*, *Blattella germanica*, *Leucophaea maderae*, and *Periplaneta americana* have proven useful in studies on JH biosynthesis by the *corpora allata* and the endocrine regulation of metamorphosis and female reproduction (*e.g.*, Stay and Tobe 1977; Lanzrein et al., 1978; Schal et al., 1997; Engelmann and Mala, 2000; Helvig et al., 2004; Marchal et al., 2013; Bellés and Santos, 2014; Zhu et al., 2020). JH III has been identified as their natural JH homolog, originally in *B. germanica* (Camps et al., 1987). Similar to other insects, presence of JH in juvenile

Abbreviations: bHLH-PAS, basic helix-loop-helix Per/Arnt/Sim; DBD, DNA-binding domain; DCM, dichloromethane; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GC, gas chromatography; Gce, Germ cell-expressed; HPLC, high-performance liquid chromatography; JH, juvenile hormone; MCPBA, m-chloroperoxybenzoic acid; Met, Methoprene-tolerant; MS, mass spectrometry; NMR, nuclear magnetic resonance; RNAi, RNA interference; Tai, Taiman; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMEDA, tetramethylethylenediamine; UAS, upstream activation sequence; UTR, untranslated region.

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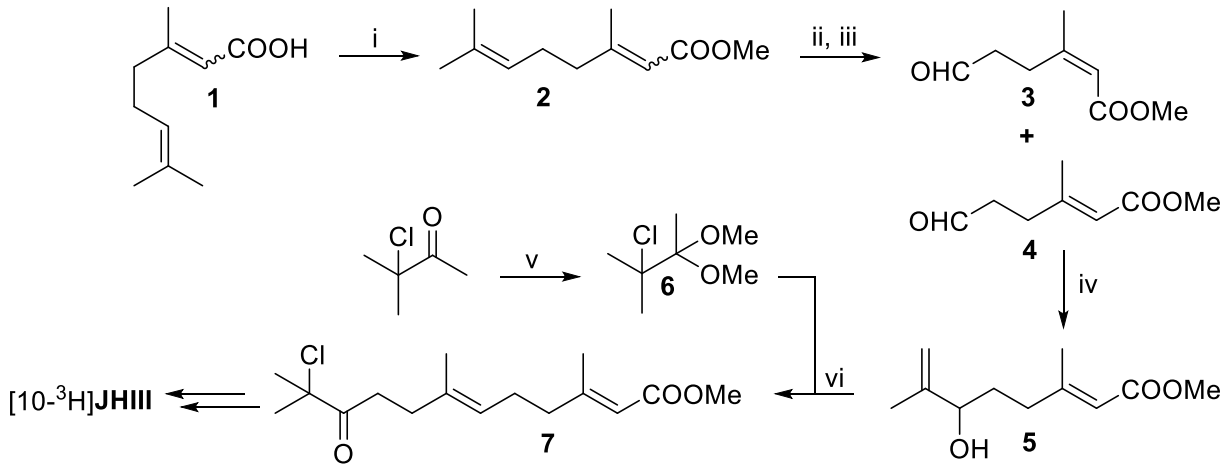
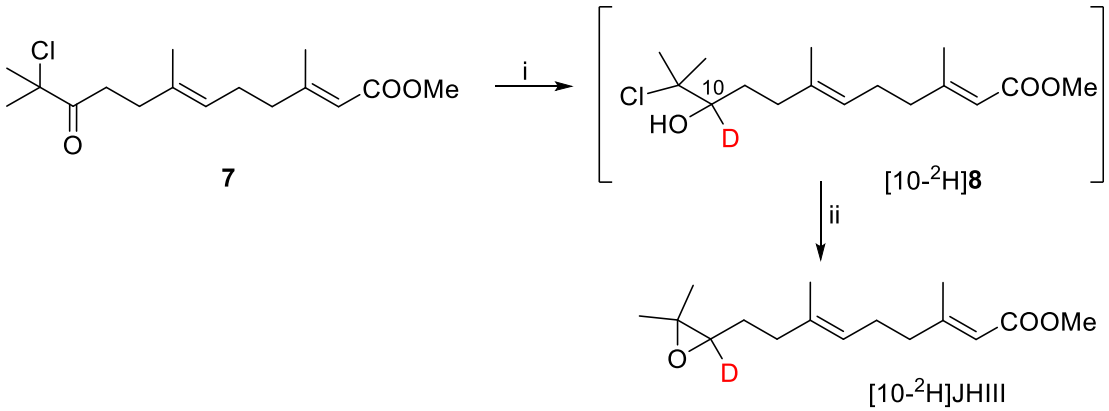


Scheme 1. Preparation of the key intermediate. Reagents and conditions: methanol, sulfuric acid, reflux, 7:3/(*E*:*Z*) ratio, 78% yield; ii) MCPBA, DCM; iii) H_5IO_6, Et_2O, **3** (11% yield over two steps), **4** (47% yield over two steps); iv) isopropenylmagnesium bromide, THF, 48% yield; v) trimethyl orthoformate, $TsOH.H_2O$, methanol, 78% yield; 2,4-dinitrophenol, toluene, 90 °C, 66% yield.



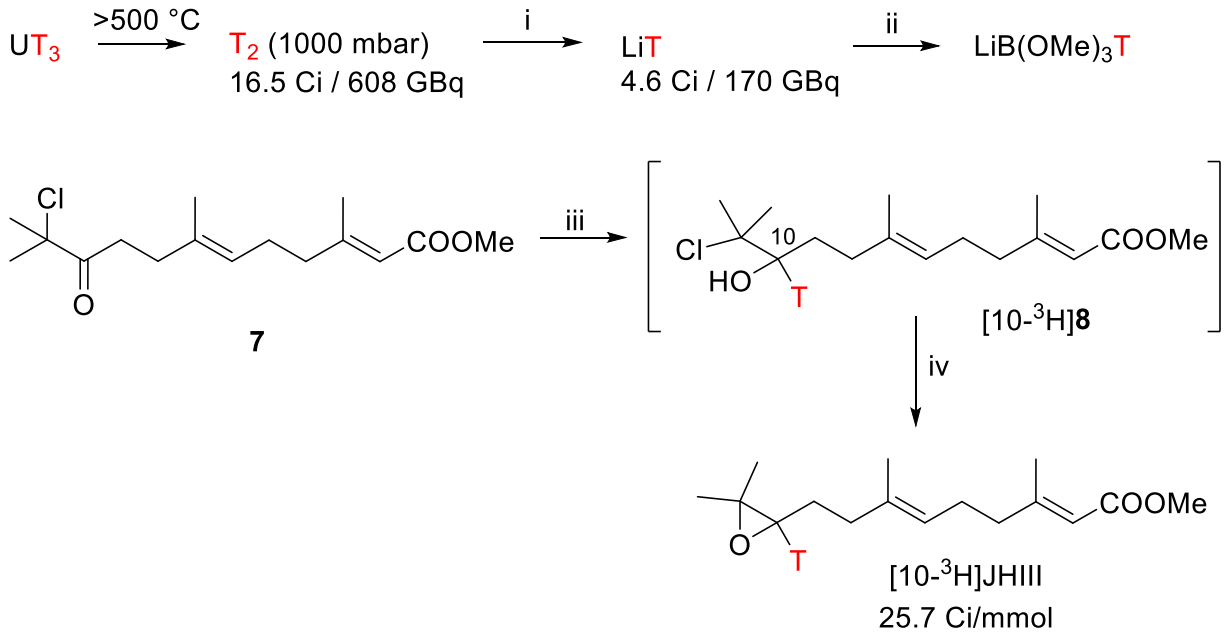
Scheme 2. Synthesis of [10-^{2}H]JH III using borodeuteride. Reagents and conditions: i) $LiB(OMe)_3D$, THF (dry); ii) K_2CO_3, methanol, 48 h.

cockroaches prevents metamorphosis until the penultimate larval instar. In adult females, JH then induces vitellogenin expression in the fat body and stimulates ovarian development.

Additional functions have been attributed to JH in regulating caste polyphenism of termites, the eusocial blattodeans, which evolved as an internal monophylum within cockroaches (Inward et al., 2007). Termites represent extreme polyphenism, manifested by castes with distinct functions. Workers, soldiers, winged reproductives, or wingless neotenic reproductives arise via caste-specific morphogenesis during post-embryonic development. JH is a long-established endocrine switch between these developmental programs (Nijhout and Wheeler, 1982; Cornette et al., 2008; Korb et al., 2012, 2021; Korb and Bellés, 2017; Oguchi et al., 2021). Soldier differentiation is informed by a marked increase in JH titer during the intermolt period and could be experimentally induced in a number of termite species using exogenous JH and many of its synthetic mimics (*e.g.*, Hrdy and Krecek, 1972; Cornette et al., 2008; Masuoka et al., 2018), suggesting potential means of controlling pest termites. Juveniles can develop into winged dispersers through a series of molts accompanied by gradually decreasing JH titer, whereas development of neotenic reproductives is triggered and/or accelerated by initially low JH titer that increases prior to a precocious molt. Positive correlation between JH level, vitellogenesis and oogenesis in reproductive females has been reported for several termite species (Elliott and Stay, 2007; Cornette et al., 2008; Maekawa et al., 2010; Saiki et al., 2015).

Blattodea have remained important models also following the establishment of the ligand-activated bHLH-PAS transcription factor Methoprene-tolerant (Met) and its partner protein Taiman (Tai) as an intracellular JH receptor (JHR) complex (Charles et al., 2011; Li et al., 2011; Kayukawa et al., 2012; Jindra et al., 2021b). The facility of systemic RNA interference (RNAi) in Blattodea has confirmed Met and Tai as *bona fide* JHR proteins governing development and reproduction of cockroaches (Lozano and Bellés, 2014; Marchal et al., 2014; Lozano et al., 2014; Zhu et al., 2020). RNAi studies in termites have likewise implicated Met in both of the major JH signaling functions, *i.e.*, as a regulator of caste morphogenesis (Masuoka et al., 2015, 2018; Sugime et al., 2019), and as a gonadotropic factor in female reproductives (Saiki et al., 2015).

Despite critical importance of JH signaling in hemimetabolous insects, neither agonist binding nor assembly of JHR complexes have been demonstrated in any representative of hemimetabolous orders. Affinities of [^{3}H]JH III binding have thus far been reported only for three holometabolous species representing the Diptera and the Coleoptera, namely for Met (Miura et al., 2005) and its paralog Gce (Jindra et al., 2015) of the fruit fly *Drosophila melanogaster*, and for Met proteins from the red flour beetle *Tribolium castaneum* (Charles et al., 2011) and the mosquito *Aedes aegypti* (Li et al., 2014). The same Met/Gce proteins have also been shown to interact in an agonist-dependent manner with the conspecific orthologs of Tai in two-hybrid assays (Miyakawa and Iguchi, 2017; Bittova et al., 2019). *In vitro* assays based on competition with [^{3}H]JH III have further allowed us to determine receptor binding affinities of unlabeled JH homologs and diverse synthetic JH agonists, such as the



Scheme 3. Radiolabeling of JH III by in-house generated borotritide. Reagents and conditions: i) *n*-BuLi, TMEDA, 2 h; ii) $B(OMe)_3$, 30 min; iii) $LiB(OMe)_3T$, THF (dry), 2 h; iv) K_2CO_3, methanol, 48 h.

juvenoid insecticides pyriproxyfen, methoprene, and fenoxycarb to JHR proteins from *T. castaneum* and *D. melanogaster* (Charles et al., 2011; Jindra et al., 2015; Bittova et al., 2019; Jindra and Bittova, 2020).

The ligand-binding assays that are critically important for studies of JH signaling have recently become unavailable due to discontinued supply of commercial radiolabeled JH preparations. Therefore, inspired by original methods, we have developed an improved protocol for synthesis of [10-^{3}H]JH III with high specific activity. Using this labeled hormone, we have been able to demonstrate high-affinity, competitive agonist binding for the recombinant Met proteins from two hemimetabolous species, *B. germanica* and *Prorhinotermes simplex*, representing cockroaches and eusocial termites, respectively.

2. Material and methods

2.1. Chemical synthesis of tritiated JH III

Racemic [10-^{3}H]JH III was prepared using a modification of established protocols (Loew et al., 1970; Werthemann and Johnson, 1970a; Baker and Schooley, 1986; Maxwell et al., 2002) and following a pilot production of [10-^{2}H]JH III through a series of reactions depicted in Schemes 1–3. The approach is detailed in the Supplementary material, including GC chromatograms (Fig. S1) and NMR spectra (Fig. S2) of the synthesis intermediates. GC, MS, HPLC, and NMR data for resulting [10-^{2}H]JH III and [10-^{3}H]JH III are given in Figs. S3–S7. Our final [10-^{3}H]JH III product had a specific activity of 25.7 Ci/mmol and was stored dissolved in toluene at −20 °C.

2.2. Met and tai genes from P. simplex and B. germanica

cDNA sequences encoding *P. simplex* Met (PsMet) and Tai (PsTai) proteins were identified in our RNAseq data (PRJNA684026, Koubová et al., 2021) using alignment with *B. germanica* protein sequences obtained from the UniProt and NCBI (Table S1). The structures of the corresponding genes were inferred from BLAST alignments of cDNA sequences onto our in-house *P. simplex* genome assembly, and exon-intron boundaries were searched manually. A similar approach was applied to examine the *tai* gene organization in *B. germanica*, where BLAST alignment with the longest available transcript (*tai* isoform A, NCBI Accession HG965205.1) (Lozano et al., 2014) was used to identify the corresponding genomic locus in the genome assembly Bger_1.1 (GCA_003018175.1) (Harrison et al., 2018). In the case of *B. germanica Met* (*BgMet*), only a partial cDNA sequence (HG965209.1) (Lozano and Bellés, 2014) was available from the NCBI. Using this sequence, we searched Bger_1.1 to identify a full-length BgMet protein sequence (PSN54894.1) and the corresponding cDNAs in the transcriptome assembly GBID00000000.1 (Table S1). These sequences were used to determine the exon-intron structure of *BgMet*, and the conceptually translated product was validated by alignment with orthologs from *D. punctata* (AIM47235.1) (Marchal et al., 2014) and *P. simplex* (this study).

The *P. simplex* and *B. germanica* Met and Tai proteins were searched for conserved domains using NCBI CD-search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple-species protein comparisons were performed with a set of arthropod sequences listed in Table S1. The sequences were aligned using Clustal Omega and phylogenetically analyzed through maximum-likelihood algorithm PhyML v3.0 with 1000 bootstrap replicates.

2.3. Cloning of Met and tai cDNAs from P. simplex and B. germanica

Specimens of *P. simplex* and *B. germanica* were obtained from cultures kept at the Institute of Entomology (Ceske Budejovice). Total RNA was isolated from adult insects and reverse transcribed using SuperScript III (ThermoFisher). Sequences encoding desired regions of the Met and Tai proteins were amplified and subcloned using PCR primers (Table S2), designed based on cDNA sequences (Table S1) that had been obtained as described in Section 2.2 above. Our RT-PCR product encompassing the entire open reading frame of BgMet encoded 952 amino acids (NCBI Accession MZ322738); its translation showed a single conflict (P208) relative to PSN54894.1, which contains serine in that position. DNA sequence encoding amino acids M1 through G517 of PsMet was optimized for human codon usage and custom synthesized (GenScript). All sequences were cloned directly to expression vectors and verified.

2.4. Ligand-binding assays

The *pK-Myc-C2* vector that contains the T7 RNA polymerase

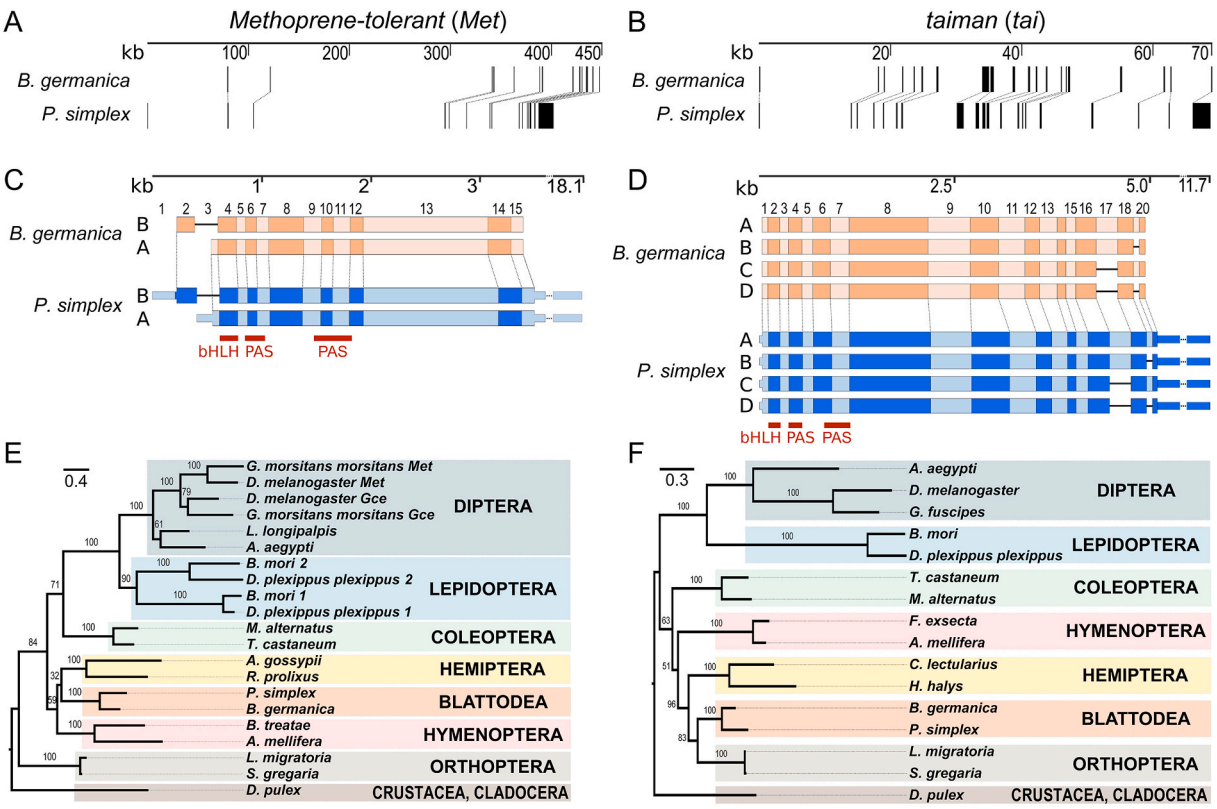


Fig. 1. Characterization of *P. simplex Met* and *tai* genes in comparison with *B. germanica* orthologs. (A, B) Blattodean *Met* and *tai* consist of multiple exons spread over large genomic loci. Orthologous exons are connected by dashed lines. (C, D) Transcript structures and splice variants. *P. simplex Met* and *tai* transcripts contain up to 14 and 20 coding exons, respectively; narrow rectangles indicate UTRs. The predicted exon-intron structure is highly conserved between the two species; exon junctions are connected by dashed lines. Sequence orthologous to exon 1 of *PsMet* could not be identified for *BgMet* in the Bger_1.1 genome assembly. Conserved bHLH and PAS domains as predicted with the NCBI CD-search tool are denoted by red bars. (E, F) Phylogenetic position of blattodean Met and Tai protein sequences within representatives of selected insect orders and *Daphnia pulex* as an outgroup. Analyzed using PhyML maximum-likelihood algorithm; branch labels show bootstrap values based on 1000 replicates. Accession numbers are provided in Table S1; for sequence alignment see Fig. S8. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

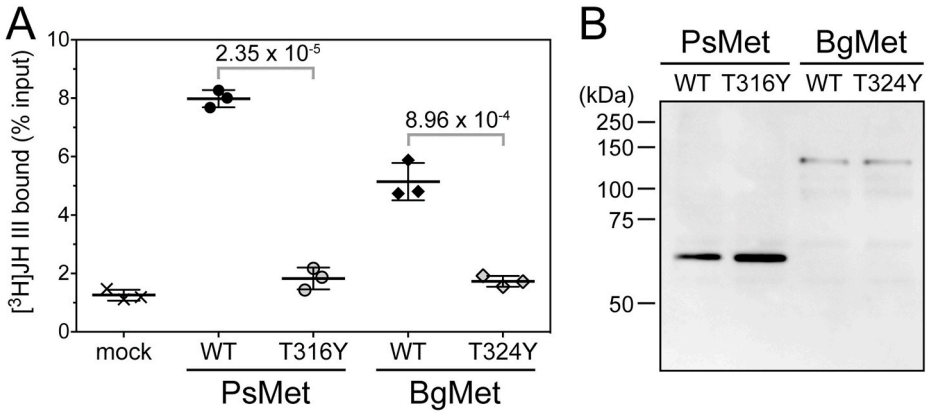


Fig. 2. Recombinant *P. simplex* and *B. germanica* Met proteins bind [^{3}H]JH III. (A) The amount of radiolabeled JH III associating with wild-type (WT), *in vitro* translated PsMet (M1-G517) and BgMet (M1-V952) proteins was significantly reduced when the critical threonine residues in the ligand-binding PAS-B domain were mutated to tyrosine (PsMet^{T316Y} and BgMet^{T324Y} variants). The numbers indicate *P*-values (unpaired *t*-test). mock, reticulocyte lysate without input DNA. Data represent mean ± S.D. (B) Immunoblotting of the WT and mutated Met proteins used in the hormone-binding assay shown in (A). The *in vitro* translated Myc-tagged products were detected using a monoclonal 9E10 anti-Myc antibody (Roche). The band sizes roughly correspond to the theoretical masses of 61.1 kDa and 109.3 kDa, calculated for the Myc-tagged PsMet (M1-G517) and BgMet (M1-V952) proteins, respectively.

promoter preceding a Myc tag sequence was used for expression of a C-terminally truncated PsMet (M1-G517) protein; BgMet was expressed either in its entirety (M1-V952) or as truncated versions BgMet (E306-V952) and BgMet (E306-P482), respectively. The N-terminally Myc-tagged Met proteins were produced *in vitro* using the TnT Quick T7 Coupled transcription/translation rabbit reticulocyte lysate system (Promega). Saturation JH-binding and competition assays were performed using the dextran-coated charcoal method (Touhara et al., 1993) as previously described (Charles et al., 2011). The rabbit reticulocyte lysate without DNA input was a background control. Briefly, the TnT products were added (in triplicates) to a reaction buffer containing increasing concentrations of [^{3}H]JH III (25.7 Ci/mmol) either alone or with excess of unlabeled JH III (Sigma-Aldrich) to assess nonspecific binding. In competition assays, a constant amount of [^{3}H]JH III was premixed with increasing concentrations of unlabeled JH III before protein addition. Following incubation for 1 h at room temperature, a

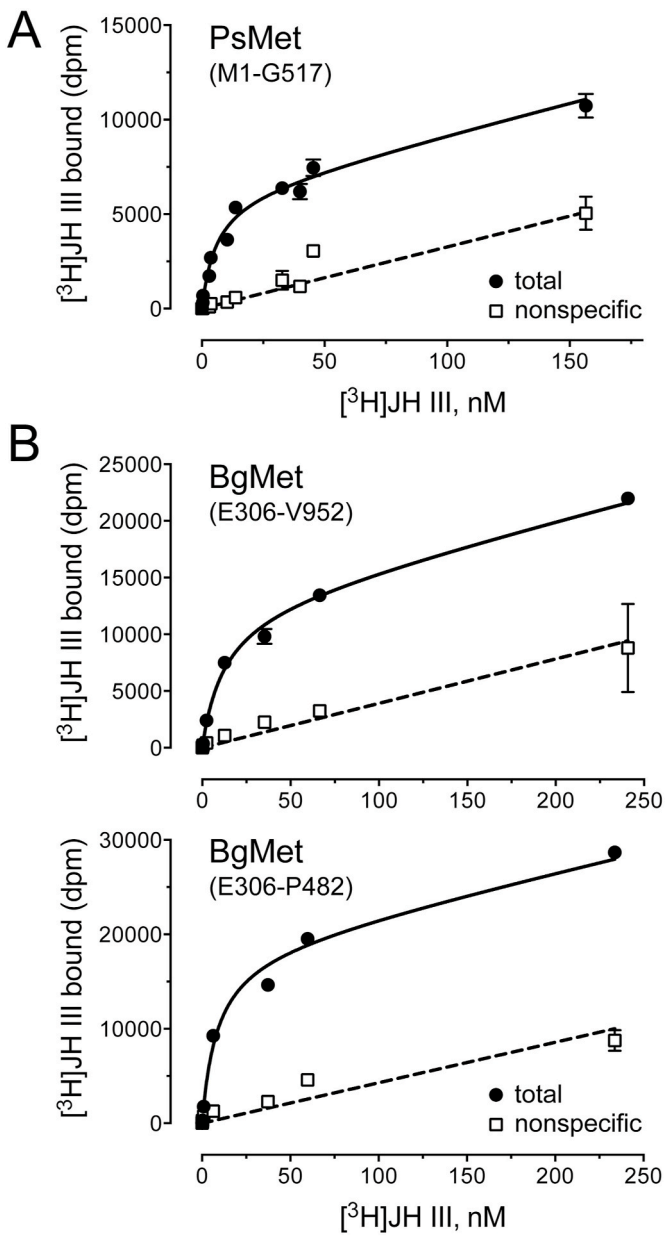


Fig. 3. High-affinity, competitive binding of [^{3}H]JH III by *P. simplex* and *B. germanica* Met proteins. The indicated fragments of PsMet (A) and BgMet (B) proteins were translated *in vitro* and incubated with increasing amounts of [^{3}H] JH III alone (total) or with excess of unlabeled JH III (nonspecific) to estimate the hormone-binding affinities (K_d values indicated in the text). The data points (mean ± S.D.) are calculated from triplicates and the experiments were repeated twice with similar results.

suspension of dextran-coated charcoal was added, and after 2 min the reaction was centrifuged for 3 min at 12,000*g*. The supernatant was collected for scintillation counting using Tri-Carb 2900 TR (PerkinElmer). Dissociation constants (K_d) were calculated using Prism 6.0 GraphPad Software (San Diego, CA) by nonlinear regression from total and nonspecific saturation binding data assuming a single binding site. Inhibition constants (K_i) were determined by nonlinear regression from competition binding data using the "one-site-fit" model with constraints given by the K_d values assessed for [^{3}H]JH III binding to the PsMet or BgMet proteins, respectively.

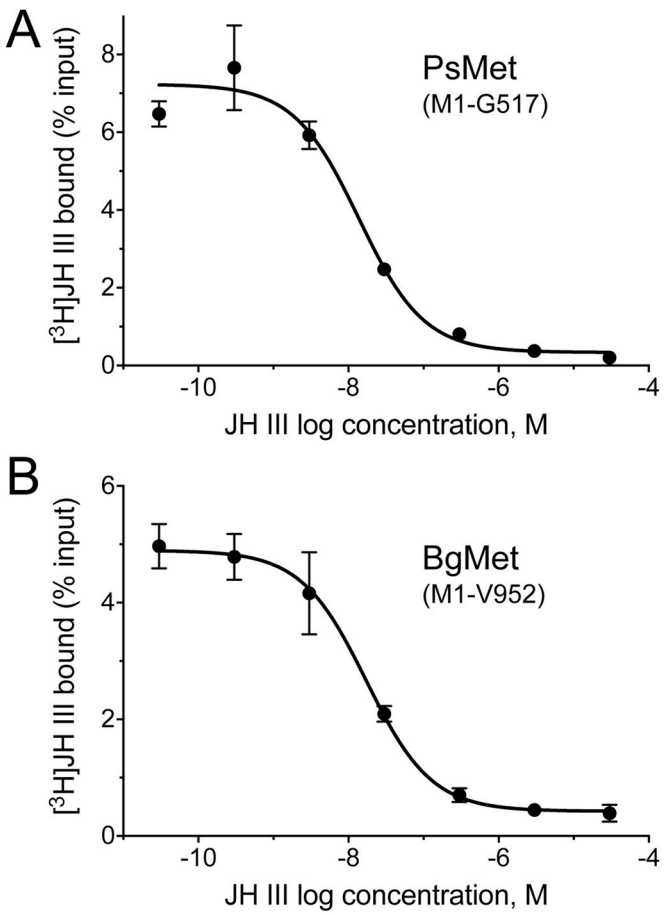


Fig. 4. Unlabeled JH III competes against [^{3}H]JH III for receptor binding. The competition assays revealed K_i values of 4.9 nM and 7.3 nM for JH III binding to PsMet (A) and BgMet (B) proteins, respectively. The data are average of three measurements; error bars represent S.D.

2.5. Site-directed mutagenesis

To generate PsMet^{T316Y} and BgMet^{T324Y} proteins bearing the single threonine-to-tyrosine substitutions in their ligand-binding PAS-B domains, the sequences encoding PsMet (M1-G517) and BgMet (M1-V952) proteins, cloned in the *pK-Myc-C2* vector, were subjected to site-directed mutagenesis using the overlap extension PCR method (Ho et al., 1989). The antiparallel primers encompassing the substitutions are shown in Table S3.

2.6. Two-hybrid assay

A two-hybrid assay for agonist-stimulated interaction between Met and Tai proteins based in mammalian cells (Miyakawa and Iguchi, 2017) was developed as follows. To express Met proteins fused with the VP16 transcription activation domain at their N-termini, DNAs encoding PsMet (M1-G517), BgMet (M1-V952), and the mutated PsMet^{T316Y} and BgMet^{T324Y} variants were cloned to the *pACT* vector (Promega). The cDNA fragments encoding the bHLH and both PAS domains of PsTai (M1-V534) or BgTai (M1-P524) were cloned to a *pDBD* vector, prepared by inserting DNA encoding amino acids M1-S147 of the Gal4 DNA-binding domain (DBD) to the *pcDNA3.1/Zeo(+)* plasmid (ThermoFisher). A reporter construct *pNL-9xUAS* was built by inserting nine repeats of the upstream activation sequence (*UAS*) before a minimal promoter in the *pNL[NLucP/minP/Hygro]* vector with NanoLuc luciferase fused to the PEST destabilization sequence (NLucP) (both Promega).

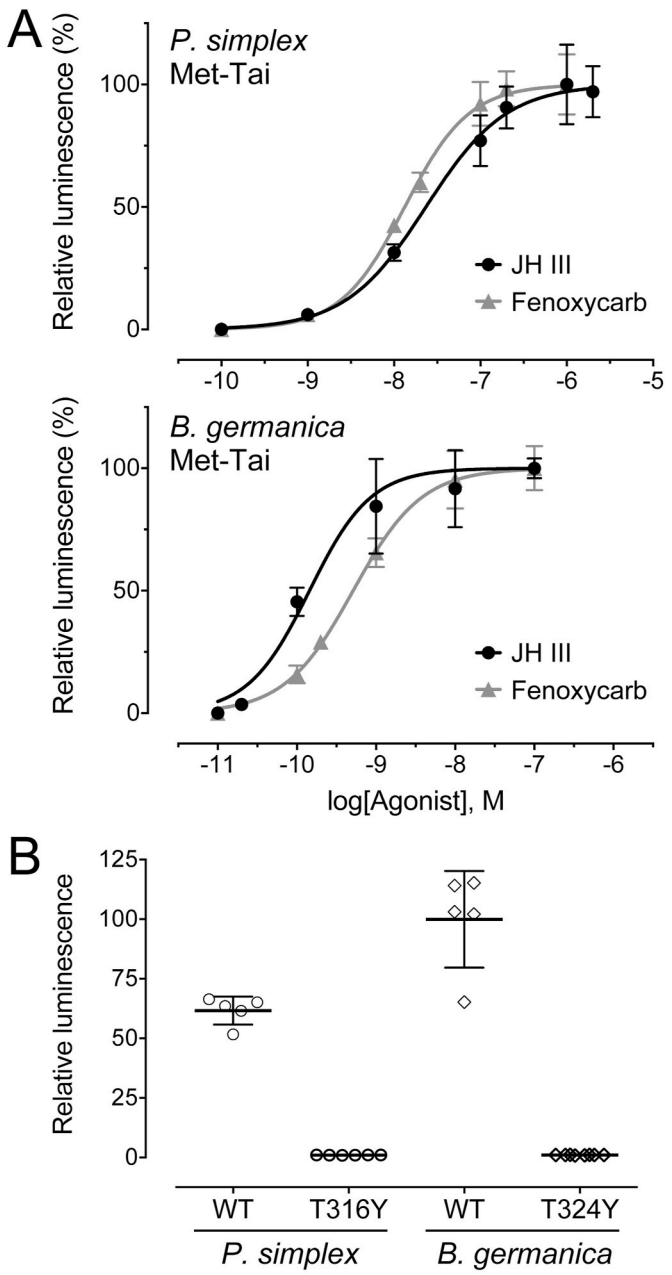


Fig. 5. JH III and fenoxycarb induce interaction between the JHR components Met and Tai from both blattodean species. (A) Two-hybrid assays with the indicated protein pairs were performed in the human HEK293 cell line. The assays were repeated three times and representative examples are shown. The data are average of three technical replicates; error bars represent S.D. (B) Interaction between *P. simplex* and *B. germanica* Met and Tai proteins was induced in the two-hybrid assay with 1 μM fenoxycarb. The Met proteins were either of wild-type sequence (WT) or were incapacitated for agonist binding by mutating the critical T316 and T324 residues, respectively. Values are average of the indicated data points; error bars represent S.D.

The assay was performed in human HEK293 cells, cultured in DMEM supplemented with 10% FBS (ThermoFisher) and penicillin/streptomycin (Bittova et al., 2019). Cells were seeded semiconfluent in poly-L-lysine coated 24-well plates, and 24 h later were transfected with the *pNL-9xUAS* reporter plasmid (220 ng/well) along with the *pACT-Met* and *pDBD-Tai* expression vectors (each 55 ng/well) using polyethylenimine. At 30 h post-transfection, cells were incubated with JH III or fenoxycarb (both Sigma-Aldrich) for 16 h, lysed, and the luciferase activity was measured using the Nano-Glo reagent (Promega) in the Orion II plate luminometer (Berthold). Vehicle alone (ethanol for JH III and DMSO for fenoxycarb) was used to determine baseline activation. EC_{50} values were calculated using Prism 6.0 GraphPad Software (San Diego, CA) by nonlinear regression (least squares ordinary fit) with the "sigmoidal dose-response (variable slope)" function.

3. Results and discussion

3.1. Chemical synthesis and effective radiolabeling of JH III

We followed a novel, straightforward synthetic route towards radiolabeled JH III that is based on a combination of previous methods of JH preparation. Our tritiation relies on the same key intermediates as reported by Baker and Schooley (1986) for the synthesis of [10-^{3}H]JH III, but differs in the applied reduction procedure and the specific activity of the product. Scheme 1 illustrates the synthesis of the key intermediate. The route was inspired by syntheses of cecropia JH 0 and JH I (Loew et al., 1970; Anderson et al., 1975) that feature a Claisen rearrangement of chloroketal (Werthemann and Johnson, 1970a, 1970b) as a cornerstone of the approach. An *E/Z* mixture of geranic acid **1** was converted to a mixture of corresponding methyl esters **2**, which were treated with MCPBA and periodic acid. This stepwise procedure yielded a separable mixture of aldehydes **3** and **4** (Henrick et al., 1972). The latter compound was reacted with isopropenylmagnesium bromide, providing allylic alcohol **5**. Chloroketal **6** was then prepared from 3-chloro-3-methylbutanone by reaction with trimethyl orthoformate (Werthemann and Johnson, 1970a, 1970b). Without purification, **6** was allowed to react with allylic alcohol **5** at 90 °C in the presence of 2,4-dinitrophenol as a mild acidic catalyst. This Claisen rearrangement furnished the key intermediate **7** in a good yield of 66%.

According to common practice in the field of radiolabeled compound synthesis, we optimized the procedures on deuterated congeners before preparing the tritiated product. To prepare a highly deuterium-enriched [^{2}H]JH III, we carried out a reduction of chloroketone **7** analogously to a procedure described in the literature (Baker and Schooley, 1986; Maxwell et al., 2002). The feasibility of selective reduction of the C-10 carbonyl moiety was proven by reaction of **7** with two equivalents of LiB(OMe)$_3{}^2$H, generated in dry THF (Scheme 2).

Reliable synthesis of the reducing agent has been reported (Zippi, 1994; Zippi et al., 1995). Briefly, LiB(OMe)$_3{}^2$H is generated from Li^{2}H by reaction with trimethoxyborate, with lithium deuteride prepared by treating an *n*-BuLi-TMEDA reaction mixture with gaseous $^2H_2$. The reduction of chloroketone **7** is relatively fast; after 90 min, a TLC analysis indicated full conversion of **7** to [^{2}H]-chlorohydrin **8**. To achieve subsequent transformation of **8** to the desired [10-^{2}H]JH III, a small amount of anhydrous K_2CO_3 in methanol was added and the mixture was vigorously stirred. The formation of oxirane was sluggish at RT; it took two days to cyclize to [10-^{2}H]JH III. The reaction proceeded with a full conversion of **8** and, fortuitously, deuterated JH III was the only product. Further experiments showed that the addition of anhydrous K_2CO_3 was a key factor. Without it, the formation of oxirane proceeded for two days with conversion into the desired [10-^{2}H]JH III as low as 20%.

The (regio)selective enrichment of the substrate with only one deuterium atom at C-10 was determined by MS and NMR analyses. ^{1}H, ^{13}C NMR, and MS spectra were combined to resolve the hydrocarbon scaffold of the product. The (2*E*)-configuration of the acrylic double bond was unambiguously confirmed by comparing GC retention times of the isolated [10-^{2}H]JH III and a known standard of unlabeled JH III (Figs. S3,S4A and S5). Both JH III and its deuterated congener provided homologous MS(EI) fragmentation patterns (Figs. S5 and S6).

Having optimized the conditions for preparation of labeled JH III, we were set to prepare [10-^{3}H]JH III (Scheme 3). For radiation safety reasons we decided to perform the reduction with borotritide and consecutive chlorohydrin **8** cyclization in a "one-pot" fashion. Therefore, upon completion of the reduction step, anhydrous K_2CO_3 was added into the

reaction mixture without any purification of [10-^{3}H]**8**.

To achieve full conversion of **7**, excess of LiB(OMe)$_3$^{3}H (1.5 eq.) was used. After three freeze-drying rounds, reduction of **7** gave a mixture of ^{3}H-labeled chlorohydrin **8** with a trace amount of [10-^{3}H]JH III. The subsequent oxirane formation was mediated through addition of anhydrous K_2CO_3 in methanol into the reaction. The cyclization yielded racemic [10-^{3}H]JH III with an activity equal to 1.8 Ci. The crude product was purified using reversed phase on semi-preparative HPLC providing pure [10-^{3}H]JH III (320 mCi) with a specific activity of 25.7 Ci/mmol (determined by MS, accounts for 0.88 tritium/molecule) and a radiochemical purity RCP >99% as determined using GC (Fig. S4A). Our yield and, importantly, the specific activity were higher than previously reported radiolabeled JH III preparations (1.4 and 20.4 Ci/mmol) (Baker and Schooley, 1986; Maxwell et al., 2002), including the formerly commercially available [10-^{3}H]JH III (10–20 Ci/mmol) (PerkinElmer).

3.2. JHR genes of P. simplex and B. germanica

Orthologs of *Met* have been cloned from *B. germanica* and *D. punctata* and shown to mediate JH effects on metamorphosis and oogenesis, respectively (Lozano and Bellés, 2014; Marchal et al., 2014). In *B. germanica*, certain alternatively spliced isoforms of *tai* also play a role during metamorphosis (Lozano et al., 2014). Termite orthologs of *Met*, represented by partial cDNAs obtained from *Reticulitermes speratus* and *Zootermopsis nevadensis*, have been implicated in vitellogenin synthesis and formation of the soldier mandibles, respectively (Saiki et al., 2015; Masuoka et al., 2015). To our knowledge, orthologs of *tai* have not been studied in termites, and detailed gene organization has not been reported for either *Met* or *tai* orthologs of any blattodean species.

P. simplex belongs to the subfamily Rhinotermitidae (also called subterranean termites), which comprises most of the important pest species. Previous research on *P. simplex*, including studies on the effect of JH mimics on caste differentiation (Wimmer et al., 2007), together with the availability of transcriptomic and genomic assemblies, make this species a relevant model. We utilized our in-house genome and transcriptome resources for homology-based mapping of gene and transcript sequences encoding the *P. simplex* Met and Tai proteins. We performed similar analyses of the JHR gene orthologs in the cockroach *B. germanica*, where we exploited local alignment of cDNA sequences obtained from public databases or published transcriptome data to Bger_1.1 genome assembly GCA_003018175.1 (Harrison et al., 2018).

The predicted exon/intron organization and transcripts of *Met* and *tai* orthologs of both species are depicted in Fig. 1. For *BgMet*, the entire coding region is confirmed by isolating a contiguous RT-PCR product (Accession MZ322738). In both blattodeans, the *Met* genes consist of 14 coding exons and occupy more than 350 kb (Fig. 1A). An exceptionally long intron 3 spanning around 200 kb in *P. simplex* also occurs in *BgMet*. Two splice variants (A and B) were identified for both *BgMet* and *PsMet* (Fig. 1C). We were unable to fully characterize the 5′UTRs including the non-coding exon 1 in *B. germanica* due to the lack of full-length transcripts and fragmentation of the Bger_1.1 genome assembly. A similar organization was also found for the cockroach and termite *tai* genes, each containing 20 coding exons and spanning around 70 kb (Fig. 1B). Like in *B. germanica* (Lozano et al., 2014), the *P. simplex* ortholog of *tai* produces at least four mRNA isoforms with alternative usage of exons 17 and 19 (Fig. 1D). All *Met* and *tai* transcripts in *P. simplex* possess long 3′UTRs spanning up to 6 kb and 14 kb, respectively (Fig. 1C and D). We were unable to confirm this feature for *B. germanica* due to the lack of full-length contigs in the available transcriptome assembly. The overall gene organization, positions of exon junctions, and sequence homology in the bHLH and in the tandem of PAS domains (Fig. S8) indicate a high degree of conservation of the JHR genes within the order. Phylogenetic positions of blattodean Met and Tai protein sequences are shown in Fig. 1E and F.

3.3. Specific, high-affinity binding of [^{3}H]JH III by blattodean Met proteins

Direct binding of JH III has thus far been reported for recombinant Met and Gce proteins from *D. melanogaster* and for the orthologs from *T. castaneum*, *A. aegypti*, and the ametabolous firebrat, *Thermobia domestica* (Zygentoma) (Miura et al., 2005; Charles et al., 2011; Li et al., 2014; Jindra et al., 2015). Our preparation of [^{3}H]JH III enabled us to test JH binding to Met proteins from the first hemimetabolous insects. The *in vitro* translated PsMet (M1-G517) and BgMet (M1-V952) proteins bound 8.0% and 5.1%, respectively, of the total [^{3}H]JH III input in the dextran-coated charcoal assay (Fig. 2A), the difference possibly reflecting lower expression of BgMet relative to PsMet (Fig. 2B). To ascertain that the binding was specific, we mutated a conserved threonine residue (Fig. S8A) in the putative ligand-binding pocket within the PAS-B domains of PsMet and BgMet. Substitution of the corresponding threonine residues with the bulkier side chain of tyrosine has been shown to preclude binding of [^{3}H]JH III by the Met/Gce proteins of *T. castaneum* (Charles et al., 2011), *A. aegypti* (Li et al., 2014), and *D. melanogaster* (Jindra et al., 2015), in the latter species blocking the ability of either Met or Gce to sustain development of the fly. Indeed, binding of [^{3}H]JH III by the mutated PsMet^{T316Y} and BgMet^{T324Y} variants was dramatically reduced relative to the wild-type controls (Fig. 2A). This reduction was not due to a failure to express the mutated proteins (Fig. 2B).

Affinities of [^{3}H]JH III binding have previously been determined through saturation ligand-binding assays for JHR proteins from three holometabolous species. The K_d values reported for Met proteins were 2.9 nM for *T. castaneum* (Charles et al., 2011), 4.4 nM for *A. aegypti* (Li et al., 2014), and 5.3 nM and 19.3 ± 4.5 nM, respectively, for the *D. melanogaster* Met and Gce paralogs (Miura et al., 2005; Jindra et al., 2015). Following the method employed in the above referenced studies, we determined that the PsMet (M1-G517) protein bound [^{3}H]JH III with a K_d of 5.7 nM (Fig. 3A), a value within the range reported for the JHR proteins from the coleopteran and dipteran species. Although BgMet (M1-V952) bound [^{3}H]JH III (Fig. 2A), it was difficult to achieve clear saturation with the full-length cockroach protein. Therefore, we expressed BgMet versions E306-V952 and E306-P482 that were truncated from one or both sides to contain the ligand-binding PAS-B domain plus or minus, respectively, the carboxyl terminus of BgMet. When expressed *in vitro*, BgMet (E306-V952) and BgMet (E306-P482) displayed K_d of 12.5 nM and 8.5 nM, respectively (Fig. 3B). This result matches the JH-binding affinity (K_d = 12.3 nM), which was reported previously for a corresponding isolated fragment of the *T. castaneum* Met protein (Charles et al., 2011).

We next tested whether binding of [^{3}H]JH III by PsMet and BgMet can be competed against with unlabeled JH III. Competition binding experiments revealed inhibition constants (K_i) equal to 4.9 nM and 7.3 nM for PsMet and BgMet, respectively (Fig. 4). These values are in a good agreement with the above K_d values for binding affinities of both proteins and with data from similar assays performed on *D. melanogaster* Gce (Bittova et al., 2019). In summary, Met proteins from both blattodean species bound our *de novo* synthesized [^{3}H]JH III with high affinities, consistent with their JH receptor function.

3.4. JH III and fenoxycarb induce interaction between blattodean Met and Tai proteins

To form a transcriptionally active heterodimer, Met interacts with another member of the bHLH-PAS family Tai upon agonist binding to Met (Li et al., 2011; Charles et al., 2011; Zhang et al., 2011; Kayukawa et al., 2012; Jindra et al., 2021a). We have adapted a cell-based two-hybrid assay (Miyakawa and Iguchi, 2017) to monitor the interaction between the two components of the blattodean JHR complexes. PsMet (M1-G517) and BgMet (M1-V952) proteins were fused with the VP16 activation domain; the Tai orthologs from *P. simplex* and *B. germanica*

fused with the Gal4 DNA-binding domain were C-terminally truncated to encompass the bHLH, PAS-A, and PAS-B domains required for binding Met. Both the PsMet-PsTai and BgMet-BgTai protein pairs interacted in response to JH III or the chemically distinct JHR agonist fenoxycarb in a dose-dependent manner (Fig. 5A), with EC_{50} ranging approximately from 0.5 to 23 nM for BgMet-BgTai and PsMet-PsTai pairs, respectively. For a reason unclear to us, relative to PsMet-PsTai the association between the *B. germanica* Met and Tai proteins was stimulated by lower agonist concentrations (Fig. 5A). Nonetheless, the interaction of both protein pairs specifically required agonist binding as no activation in the two-hybrid assay occurred with the mutated $PsMet^{T316Y}$ and $BgMet^{T324Y}$ variants even at a high fenoxycarb concentration (Fig. 5B).

4. Conclusions

This study addresses notable gaps relevant to our understanding of JH signaling. First, there is the current lapse of availability of a radiolabeled JH, which is an essential tool for determining properties of JH receptors or any potential JH-binding proteins. Straightforward reduction of the carbonyl group in the chloroketone precursor using lithium trimethoxyborodeuteride afforded a high yield of [10-^{2}H]JH III (up to 95%) with deuterium enrichment of over 95% at the sp^3 carbon (C-10). The final tritiation under optimized conditions provided [10-^{3}H]JH III in an isolated yield of 320 mCi and a specific activity of 25.7 Ci/mmol, exceeding specific activities of previous [10-^{3}H]JH III preparations. The final product proved active in high-affinity, competitive binding to insect JHR proteins, thus offering itself to future studies.

The other outcome of this work addresses the lack of knowledge on JHR protein function in hemimetabolous insects. To this end, we provide a systematic description of blattodean genes encoding JHR components. Successful synthesis of [10-^{3}H]JH III enabled us to determine the ligand-binding properties of the recombinant JHR proteins. The JHR proteins we chose to analyze originate from a cockroach and a eusocial termite, *i.e.*, species representing two lineages of an important insect order, in which JH executes a general reproductive function and plays an additional specific role in social caste differentiation.

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Appendix A. Supplementary data

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