Ecdysterone Enhances Muscle Insulin Signaling by Modulating Acylcarnitine Profile and Mitochondrial Oxidative Phosphorylation Complexes in Mice Fed a High-Fat Diet

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OBJECTIVE—To investigate the underlying mechanisms of ecdysterone (Ecdy) antidiabetic action in the diet-induced obesity animal model.

RESEARCH DESIGN AND METHODS—Mice fed a high-fat diet (HFD) were treated with or without Ecdy at a low (Ecdy L) or high (Ecdy H) dose (25 or 50 mg/kg body wt) for 12 weeks. The effects of Ecdy on food intake, energy expenditure, glucose metabolism, insulin signaling, muscle lipid profile, and mitochondrial oxidative phosphorylation (OxPhos) complexes were evaluated.

RESULTS—At the end of the study, there were no differences in body weight, food intake, and energy expenditure between HFD groups with or without a diet supplemented with Ecdy L, except body weight was significantly lower in the Ecdy H group than in the HFD group (P < 0.05). Plasma glucose, insulin levels, and values of the homeostasis model assessment of insulin resistance in the Ecdy H group were significantly lower than in the HFD group (P < 0.01). Insulin-stimulated phosphorylation of insulin receptor substrate 1 and Akt1 in muscle was significantly increased in the Ecdy H group (P < 0.001) relative to the HFD group (P < 0.01). Moreover, muscle fatty acylcarnitine profile showed the HFD significantly increased muscle lipid contents compared with a low-fat diet (P < 0.01) but reduced OxPhos complex abundance. Ecdy reversed the effects of HFD-induced lipid accumulation by significantly increasing the abundance of mitochondrial OxPhos complex protein in muscle.

CONCLUSIONS—We observed that Ecdy improved glucose metabolism and enhanced muscle insulin signaling by altering the acylcarnitine profile and increasing mitochondrial OxPhos complexes in HFD-fed mice. Our study suggests that these effects of Ecdy are dose-dependent.
reflection of mitochondrial metabolism. Research in recent years has shown that acylcarnitines are important biomarkers for the diagnosis of mitochondrial fatty acid β-oxidation and branched-chain amino acid oxidation disorders, assuming they reflect the potentially toxic acyl-CoA species, accumulating intramitochondrially upstream of the enzyme block (25). Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is considered to be a major regulator of mitochondrial biogenesis (26). In addition, carnitine palmitoyltransferase 1 (CPT1) and uncoupling proteins 3 (UCP3) are important in regulating cellular fuel metabolism and are increasingly implicated in a range of pathophysiologic processes, including obesity, insulin resistance, and diabetes (27,28).

To date, knowledge is limited about the effects of Ecdy on the muscle insulin-signaling pathway, acylcarnitine content, and mitochondrial oxidative phosphorylation (OxPhos) complexes in animal models. We hypothesize that the mechanism by which Ecdy lowers glucose levels in insulin-resistant conditions may result from enhancing insulin signaling of tissues by altering muscle fatty acylcarnitine content and mitochondrial protein expression. To test our hypothesis, we measured the effects of Ecdy on body weight, fasting glucose, insulin levels, the muscle insulin-signaling pathway, acylcarnitine content, and mitochondrial OxPhos complex proteins in mice fed a high-fat diet (HFD).

**RESEARCH DESIGN AND METHODS**

**Reagents and chemicals.** Ecdy (98% of purity) was purchased from Bosche Inc. (New Brunswick, NJ). Human insulin was obtained from Eli Lilly (Indianapolis, IN). Anti-insulin receptor subunit 1 and 2 (IRS-1, IRS-2) polyclonal antibodies were obtained from Santa Cruz Bioreagents (Golden, CO). c-Actin antibody was obtained from Affinity Bioreagents (Golden, CO). IR, β- and CPT1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were ordered from Sigma Chemicals (St. Louis, MO).

**Animals.** Twenty-six male 4-week-old C57BL/6 J mice (Jackson Laboratory, Bar Harbor, ME) were housed one per cage with ad libitum access to rodent chow and water for a 2-week acclimation period under specific pathogen-free conditions and a light-dark cycle. During the 12-week study, animals were randomly divided into four treatment groups: normal chow control (control, n = 6), HFD group (n = 6), a low-dose Ecdy group (Ecdy L, n = 7) receiving Ecdy at a dose of 25 mg/kg body wt, and a high-dose Ecdy group (Ecdy H, n = 7) receiving 50 mg/kg body wt. Ecdy was incorporated into the HFD (wt/wt). The HFD contains 58% kcal from fat (D12331) and low-fat diet (LFD) contains 10% kcal from fat (D12250B); both diets were purchased from Research Diets Inc. (New Brunswick, NJ). The diets were stored at −20°C. All animal experiments were performed according to the protocol approved by Institutional Animal Care and Use Committee of Pennington Biomedical Research Center.

**Body weight and food intake.** Body weight and food intake were measured weekly to monitor changes. Fasting (4-h) plasma glucose, insulin, and body composition were determined at weeks 0, 6, and 12. A metabolic chamber study was performed at week 8. At the end of the study, mice in each group were randomly divided into basal and insulin-stimulated subgroups, and the vastus lateralis muscles were dissected at basal or insulin-stimulated conditions (at 10 min after an intraperitoneal insulin injection at a dose of 2 units/kg body wt) during necropsy. Plasma, skeletal muscle, liver, and other tissues also were collected, quickly placed in a liquid nitrogen container, and stored at −80°C for later analysis.

**Blood chemistry and hormone analysis.** After a 4-h fast, blood samples were collected from the orbital sinus of unconscious mice induced by CO2 inhalation. Plasma glucose level was measured by a colorimetric hexokinase glucose assay (Sigma Diagnostics, St. Louis, MO). Plasma insulin level was determined by an ultrasensitive rat insulin ELISA kit (Crystal Chem, Downers Grove, IL).

**Body composition measurement.** Body composition was measured by using a Minispec TD-NMR Spectrometer (Bruker Optics, The Woodlands, TX) (29). Total fat mass (FM) and free fat mass (FFM) were recorded.

**Intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test.** The effects of Ecdy and the diets on insulin and glucose parameters were determined by an intraperitoneal glucose tolerance test (IPGTT) at week 10 (29), and an intraperitoneal insulin tolerance test (IPITT) was conducted by intraperitoneal injection of 1 unit/kg body wt insulin (Sigma) at week 11. Whole blood glucose was measured from a sample collected from the tail vein using the FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ) at time 0 (baseline), and at 15, 30, 60, and 120 min after glucose or insulin injections.

**Metabolomics analysis.** At 12 weeks, vastus lateralis muscle was collected from mice and snap-frozen. Muscle extracts were prepared for HPLC analysis of fatty acylcarnitine by tandem mass spectrometry at the School of Veterinary Medicine at the Louisiana State University, as previously described (30).

**Western blot analysis.** Muscle tissue lysates were prepared by dissection and homogenized in buffer A (25 mmol/L HEPES [pH 7.4], 1% Nonidet P-40 [NP-40], 137 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 μg/mL pepstatin, and 5 μg/mL leupeptin) using a PRO 200 homogenizer (PRO Scientific, Oxford, CT). The samples were centrifuged at 14,000g for 20 min at 4°C, and protein concentrations of the supernatant were determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Supernatants (50 μg) were resolved by SDS-PAGE for immunoblotting. Protein abundance was detected with antibodies against IRS-1, IRS-2, anti-phospho-IRS-1 (Tyr1161), p55 of PI 3K, phospho-Akt (Ser473), 4G10, CPT1, PGC-1α, and β-actin (1:1000 dilution) using Chemiluminescence Reagent Plus (PerkinElmer Life Science, Boston, MA) and quantified using a densitometer. All results were normalized by β-actin levels (28).

**Mitochondria isolation and OxPhos complexes measurement in muscle tissues.** Mitochondria isolation and measurement of total OxPhos complexes were performed with mitochondrial isolation and rodent total OxPhos complexes detection kits according to the manufacturer's instruction (MitoSciences Inc., Eugene, OR). Briefly, mitochondria were isolated from muscle tissues, and the mitochondrial protein (15 μg) was subjected to a 5–20% gradient SDS-PAGE and then transferred to nitrocellulose membrane. OxPhos complexes 1 (NADH-oxidase Q reductase), II (succinate-coenzyme Q reductase), III (coenzyme Q-cytochrome C reductase), IV (cytochrome C oxidase), and V (ATP synthase) were detected with a monoclonal antibody cocktail (MitoSciences).

**Statistical analysis.** All data are expressed as mean ± SEM. Comparisons of groups were done by two-sided t test or ANOVA for experiments with more than two subgroups. A P value < 0.05 was considered significant.

**RESULTS**

**Effects of Ecdy and HFD on net body weight gain and food intake in mice.** Mice fed the HFD had significantly increased body weight compared with mice fed the LFD. The body weight in the Ecdy L group was similar to the HFD group, but was slightly lower in the Ecdy H group than in the HFD and Ecdy L groups (Fig. 1A). At the end of the study, the net body weight gains were 18.9 ± 1.7 g in HF, 19.8 ± 1.9 g in Ecdy L, 16.1 ± 1.4 g in Ecdy H, and 6.5 ± 0.4 g in the LFD group. Net body weight gain was not significantly different between HFD and Ecdy L groups, but the gain was significantly lower in the Ecdy H and LFD groups (P < 0.05) than in the HFD groups (P < 0.001). Food intake in the HFD, Ecdy L, and Ecdy H animals were significantly higher than in the LFD animals (P < 0.001). However, food intake did not differ among the HFD, Ecdy L, and Ecdy H animals.

**Effects of Ecdy on body composition and insulin sensitivity in mice.** Body composition results showed the HFD greatly increased FM and FFM in all three groups, with or without Ecdy, compared with the LFD group at 6 (P < 0.05) and 12 weeks (P < 0.001). FM was higher in the Ecdy L group than in the Ecdy H and LFD groups, but the differences among these three groups were not significant. The FFM in Ecdy L and Ecdy H mice was slightly lower than in the HFD group. FFM in the LFD group was greatly lower than in the HF and HFD and Ecdy L groups (P < 0.001; Fig. 1C and D). Body composition in the LFD groups did not significantly change, but FM significantly increased and FFM significantly decreased in all HFD groups, with or
without Ecdy, FM and FFM levels were no different among HFD groups.

Ecdy supplementation decreased fasting plasma glucose, insulin, and homeostasis model assessment of insulin resistance (HOMA-IR) in the HFD-fed mice. Fasting plasma glucose levels were not significantly increased in the HFD-fed mice until week 12, but plasma insulin levels and the HOMA index dramatically increased at week 6. Plasma insulin concentration and HOMA-IR values were significantly lower in the Ecdy H mice than in the HFD mice, but not in the Ecdy L animals (Fig. 2A–C). IPGTT and IPITT results revealed a similar trend in the Ecdy L and HFD groups (Fig. 3).

**Ecdy enhanced muscle insulin-signaling in mice.** Immunoblotting analysis showed insulin-stimulated muscle phosphorylation of IRS-1 and Akt1 in the HFD and in Ecdy L groups was significantly lower than in the LFD group ($P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively). Insulin-stimulated muscle phosphorylation of IRS-1 and Akt1 was significantly increased in the Ecdy L and Ecdy H groups than in the HFD group ($P < 0.001$ and $P < 0.01$, respectively). Basal phosphorylation of AS160 was significantly lower in the HFD group than in the LFD group.

**Ecdy reduced acylcarnitine accumulation in muscle.** Fatty acylcarnitine profile showed the HFD significantly increased eight acylcarnitines, including C5, C8-DC, C10-1, C12-OH, C14-2, C14-OH, C18-2, and C18-1 acylcarnitine content in muscle compared with the LFD animals ($P < 0.01$ and $P < 0.001$). Contents of nine acylcarnitines,
including C5, C8-1, C8-DC, C10-1, C12-OH, C14-OH, C16, C18-2, and C18-1 in muscle were substantially reduced in the Ecdy H group relative to the HFD group ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; Fig. 5A and B). However, Ecdy L only significantly decreased some of them, including C5, C14-2, C14-1, C14-OH, C18-2, and C18-1, but increased C18-1-OH contents compared with the HFD group. Ecdy altered muscle mitochondria and related protein content. Because PGC-1α and related proteins are specifically involved in the maintenance of mitochondrial function, respiration, and content in skeletal muscle (24), we measured effects of HFD and Ecdy on PGC-1α, UCP3, and CPT1 protein content in mice muscle. Muscle PGC-1α and CPT1 proteins were modestly lower in the HFD group (a decrease of ~27 and 25%), and were higher in the Ecdy H group than in the LFD group (increase of 12 and 10%). UCP3 protein concentration was significantly lower in the HFD and Ecdy L groups than in the LFD group ($P < 0.01$). However, the concentration of these proteins was significantly higher in the Ecdy H group than in the HFD or Ecdy L groups. The protein concentrations of PGC-1α, UCP-3, and CPT1 increased 52, 147, and 41%, respectively, in Ecdy H mice relative to the HFD mice, but these protein concentrations were not significantly different between the Ecdy H and LFD mice (Fig. 6).

To examine if Ecdy affects muscle mitochondrial respiratory chain complexes, we measured OxPhos complexes in the mitochondria purified from mice muscle. The HFD significantly reduced muscle mitochondrial content of all five OxPhos complex proteins compared with the LFD ($P < 0.01$ and $P < 0.001$), whereas Ecdy treatment substantially increased expression of mitochondrial OxPhos complexes, especially in the Ecdy H group compared with the HFD groups ($P < 0.05$ and $P < 0.001$; Fig. 7).

**DISCUSSION**

The chemical structure of Ecdys is based on the C-27 cholesterol skeleton; however, they differ from the vertebrate steroids in their polarity and bulkiness (Fig. 7).
fact, Ecdys are structurally similar or identical to insect molting hormones and produce a range of effects in mammals, including increasing growth and physical performance. An earlier animal study showed Ecdy possessed anabolic effects with no androgenic effects compared with methandrostenolone (Dianabol) (5,10).

An important aspect of the Ecdys is their low toxicity in mammals. The median lethal dose of Ecdy in rodents is 6.4 g/kg body wt for intraperitoneal injection and >9 g/kg body wt when given orally (31). Our study showed that the high dose of Ecdy significantly reduced body weight after 11 weeks (Fig. 1), without significantly altering food intake. The higher doses of Ecdy were used in this study because the Ecdy was incorporated into the HFD instead being administered by gavage (4). Ecdy was able to exert a glucose-lowering effect in hepatocytes that was insulin-independent, but an in vitro study showed it had no effect on insulin release (32).

Earlier observation reported Ecdy enhanced insulin sensitivity in the rat insulin-resistance model induced by injections of hydrocortisone (16). Recent studies illustrated that long-term Ecdy administration protected rats from streptozotocin-induced hyperglycemia and reduced obesity and insulin resistance in the diet-induced obesity mouse model (17,18). We demonstrated that Ecdy improved glucose metabolism and insulin sensitivity in mice fed a HFD by enhancing insulin signaling, altering the acylcarnitine profile, and modulating the content of mitochondrial OxPhos complexes in muscle tissues.

The major effects of Ecdy were to reduce plasma glucose and insulin levels and increase insulin sensitivity and insulin signaling in a dose-dependent manner in HFD-fed

**FIG. 3.** IPGTT and IPITT measurements in mice were performed after 4-h fasting at week 10 or week 11, respectively. Data show IPGTT (A and B) and IPITT (C) were significantly higher in the HFD, Ecdy L, and Ecdy H groups than in the LFD group and were significantly lower in Ecdy H than in HFD animals. Mean ± SEM (n = 6–7/group). **P < 0.01 and ***P < 0.001. #P < 0.05 for Ecdy H vs. HFD group, respectively.
mice. Body weights were significantly lower in the LFD group than in the other three groups and were significantly lower in the Ecdy H group (at the last 2 weeks) than in the Ecdy L and HFD groups. Body weight was not significantly different between Ecdy L and HFD mice. Plasma glucose, insulin levels, and HOMA values in the Ecdy H animals were significantly lower than in the Ecdy L and the HFD groups, but these parameters were not significantly different between the Ecdy L and HFD groups. This indicates that the higher Ecdy dose is required to achieve significant improvement of glucose metabolism in the insulin-resistant state, such as diet-induced obesity. However, whether Ecdy altering satiety is dosage-dependent needs to be further investigated.

We also observed that Ecdy supplementation altered muscle cellular signaling and acylcarnitine profile in mice fed a HFD. A negative relationship has been well documented between muscle lipid contents and insulin signaling (33). Several factors may contribute to increased lipid deposition in muscle. An increase in fatty acid uptake without any change in oxidation could lead to cytosolic lipid accumulation (34). Conversely, an impaired ability to use fat as a fuel source because of reduced activity of enzymes of oxidative metabolism and fatty acid utilization could also result in increased cytosolic lipids (34–36). Prominent among these so-called lipotoxic mediators are signaling molecules, such as long chain acyl-CoAs, ceramides, and diacylglycerols, each of which is thought to engage serine kinases that disrupt the insulin-signaling cascade, thereby causing insulin resistance (37). The relative palmitic acid concentration in muscle, diacylglycerols, and the serum palmitoylcarnitine concentration were higher in healthy subjects fed the high-palmitic acid diet (37).

Although we did not measure ceramides and diacylglycerols in current study, HFD reduced short-chain fatty acid, such as C4-DC and C5-1, and increased long-chain fatty acid in the muscle tissues, whereas Ecdy attenuated or reversed the effects of the HFD on muscle acylcarnitines. Our acylcarnitine profile data were similar to recent findings that acylcarnitine profiles of PPAR-α−/− mice revealed a two- to fourfold accumulation of long-chain

FIG. 4. Measurement of insulin signal transduction pathway protein is shown in skeletal muscle of mice with or without insulin stimulation (intraperitoneal injection of 2 units/kg body wt for 10 min). Western blotting data show phosphorylation of IRS-1, IR-β, Akt1, and AS160, which was normalized by the corresponding protein. Mean ± SEM (n = 6–7/group), *P < 0.05, **P < 0.01, and ***P < 0.001 for HFD, Ecdy L, or Ecdy H vs. LFD control. #P < 0.05 and ##P < 0.01 for HFD vs. Ecdy L or Ecdy H, respectively.
species in the plasma, whereas short-chain species were reduced by as much as 69% in plasma, liver, and skeletal muscle compared with PPAR-α+/+ animals (38).

Defects in skeletal muscle fat oxidation have been implicated as a driving factor contributing to systemic lipid imbalance, which leads to intramitochondrial metabolite accumulation, mitochondrial stress, and cellular insulin resistance (39). The role of fatty acid oxidation in regulating insulin sensitivity is controversial, however, and the mechanisms remain unresolved. A recent study reported that body and liver fat mass rather than muscle mitochondrial function determines glucose metabolism in women with a history of gestational diabetes (40). Moreover, other studies in mice show that increased mitochondrial OxPhos is associated with obesity and insulin resistance (41–43). Whether the effects of Ecdy on acylcarnitine profile and the expression of mitochondrial OxPhos complexes in the liver are similar to those in muscle remain to be further investigated.

As evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents, Turner et al. (44) observed high lipid availability does not lead to intramuscular lipid accumulation and insulin resistance in rodents. In contrast to their findings, we demonstrated that the HFD caused insulin resistance and reduced mitochondrial proteins in mice muscle. The different results between the studies may be explained by the different HFD used (both from Research Diets Inc., New Brunswick, NJ): we fed mice with the D12331 diet, containing 58% of energy from fat (93.3% saturated fat) without dietary fiber, whereas Turner et al. used diet D12451, containing 4% of fibers from fat (only 3.6% saturated fat) with 5% dietary fiber-cellulose.

Overexpression of CPT1 in skeletal muscle in vivo increases fatty acid oxidation, reduces triacylglycerol esterification, and improves HFD-induced insulin resistance (27). UCP3 was observed to protect mitochondria against intramitochondrial nonesterified fatty acids. UCP3 expression was significantly impaired muscle insulin signaling and reduced OxPhos complexes compared with the LFD, whereas Ecdy supplementation significantly increased muscle PGC-1α, CPT1, UCP3, and OxPhos complex protein expression compared with the HFD group.

FIG. 5. Ecdy reduced fatty acylcarnitine content in mice muscle. Muscle fatty acylcarnitine profiles were obtained by tandem mass spectrometry from mice fed with a LFD (n = 6), HFD (n = 7), and Ecdy L and Ecdy H (n = 7). The effect of the HFD and Ecdy on short- and medium-chain (A) and long-chain fatty acylcarnitine (B) was assessed by ANOVA, as described in RESEARCH DESIGN AND METHODS for each acylcarnitine subtype. C4-DC, succinyl carnitine; C5-1, tiglylcarnitine; C5, isovalerylcarnitine/methylbutyrylcarnitine; C8:1, oleoylcarnitine; C10-1, decenoylcarnitine; 12:OH, 3-hydroxydecanooylcarnitine; C14-2, tetradeциenoylcarnitine; C14-1, tetradeconoylcarnitine; C14-OH, 3-hydroxytetradecanoyl-carnitine; C16, palmitoylcarnitine; C18-1, oleoylcarnitine; and C18-1-OH, 3-hydroxyesteroylcarnitine. Mean ± SEM. **P < 0.01 and ***P < 0.001, respectively, HFD or Ecdy vs. LFD. #P < 0.05, ##P < 0.01, Ecdy L and Ecdy H vs. HFD.
FIG. 6. Effects of HFD and Ecdy are shown on muscle UCP3, CPT 1, and PGC-1α protein abundance (A) as well as on mitochondrial OxPhos complex proteins (B) in mice. UCP3, CPT 1, and PGC-1α of muscle lysates were measured by using Western blotting, and results were normalized by β-actin. Muscle mitochondria were isolated by a kit and subjected to a 5–20% gradient SDS-PAGE. After transfer to a nitrocellulose membrane, OxPhos complexes I, II, III, IV, and V were detected with anti-OxPhos complexes monoclonal antibody cocktail. Data are presented as mean ± SEM (n = 6–7/group), *P < 0.05, **P < 0.01, and ***P < 0.001 for HFD, Ecdy L, or Ecdy H vs. LFD control. #P < 0.05 and ##P < 0.01 for Ecdy L or Ecdy H vs. HFD, respectively.
These findings support the concept that acylcarnitine profiles may be an important biomarker for the diagnosis of mitochondrial fatty acid $\beta$-oxidation (25). However, our current study cannot directly address correlations among muscle acylcarnitine content, insulin signaling, and mitochondrial OxPhos complex expression in Ecdy-treated mice because changes in metabolic parameters and fat content were measured over the course of the study, while mitochondrial proteins were assessed.

Earlier evidence showed that depletion of mitochondrial DNA causes impaired glucose utilization and insulin resistance in L6 GLUT4myc myocytes (45). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle mitochondrial dysfunction may play a key role in insulin resistance in type 2 diabetes and obesity (48). Our data further support the notion that skeletal muscle mitochondrial dysfunction may play a key role in insulin resistance in diet-induced obesity animal models and that Ecdy enhances insulin signaling by increasing mitochondrial complex protein expression (45–48).

In conclusion, our study shows that Ecdy improves glucose metabolism and enhances muscle insulin signaling in the HFD-fed mice by a reduction of acylcarnitine accumulation and an increase of mitochondrial OxPhos complexes. It suggests that these effects of Ecdy on insulin signaling, acylcarnitine profile, and mitochondrial OxPhos complexes appear to be dose-dependent.

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Z.Q.W. designed the study, wrote and edited the manuscript, and had primary responsibility for the final content. Y.Y. and X.H.Z. researched data. D.R. and W.T.C. reviewed the manuscript. All authors read and approved the final manuscript.

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# FIG. 7. A: Chemical structure of ecdysterone is shown. Molecular formula: C$_{21}$H$_{34}$O$_{7}$; molecular weight: 480.63. B: Structure of dihydrotestosterone (DHT) is shown. Molecular weight: 290.44. The structure of testosterone closely resembles that of DHT, a hormone of key importance in the development of male sex organs.


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