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16α-IODO-TESTOSTERONE: CHEMICAL SYNTHESIS AND EVALUATION AS A POTENTIAL RADIOPHARMACEUTICAL

ELIZABETH K. SYMES,* W. F. COULSON,* R. D. FARRANT†‡ and E. J. G. MILROY§

*Courtauld Institute of Biochemistry and §Department of Urology, Middlesex Hospital Medical School, Mortimer Street, London, W1P 7PN; †Department of Chemistry, Westfield College, University of London, Kidderpore Avenue, Hampstead, London, NW3 7ST, U.K.

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Abstract—The chemical synthesis and characterization, including ^{1}H NMR, of 16α-iodo-androstenedione and 16α-iodo-testosterone are described. Each has been synthesized with ^{125}I and tested in rats *in vivo* for accumulation in androgen dependent tissues over a 24 hr time course. Neither compound was accumulated in prostate against the blood gradient of normal or 24 hr castrate animals. The metabolism, subcellular distribution and binding of 16α-[^{125}I]iodo-testosterone to protein in prostate has also been examined. By comparison with data obtained after the administration of [^{3}H]testosterone we conclude that the failure of this iodinated androgen to accumulate in androgen dependent tissues arises because of its low binding affinity for receptor protein.

In the field of external gamma scintigraphy the sophistication of the instrumentation at the present time exceeds that of the radiopharmaceuticals available. However, in the case of breast and prostate cancers, their sex hormone receptor content offers the possibility of developing radiopharmaceuticals which would be of value for imaging soft tissue deposits and would in addition indicate appropriate therapy [1].

The growth of many breast and prostate tumours is sex hormone dependent and can be arrested by withdrawal of the hormone stimulus. The oestrogen receptor status of breast tumours has been shown to correlate well with remission after oestrogen ablation or anti-oestrogen therapy [2]. Similar though less convincing correlations have been reported for the androgen receptor content and response to therapy of prostate tumours [3, 4]. Thus a knowledge of tumour receptor content is clinically useful.

Eckelman *et al.* [1] suggested that radiolabelled oestrogens with high receptor binding affinity should be accumulated by oestrogen receptor containing breast tumours and metastases and, therefore, permit the imaging of these tissues by external gamma scintigraphy. This technique is non-invasive and would provide information about both the spread of the disease and the receptor status of the tumour. However, it depends on the existence of suitable gamma emitting radiopharmaceuticals that are concentrated selectively in the target tissue, with tissue to background ratios of at least 5:1 [5].

In 1979 Hochberg [6] described the synthesis of 16α-iodo-oestradiol. He demonstrated that its receptor binding affinity was equal to that of oestradiol and that it was oestrogenic *in vivo* [7]. Both the 16α-^{125}I and 16α^{77}Br derivatives have also been shown to be accumulated by oestrogen sensitive tissues of the rat such as uterus and DMBA mammary tumours [8, 9] and the 16α-^{77}Br derivative [10] and 16α-^{131}I derivative [11] have been shown to accumulate in some oestrogen receptor positive breast tumours.

A similar gamma-emitting androgen analogue that binds to androgen receptor and accumulates in prostatic tissue has so far not been synthesized. Hoyte *et al.* [12] have synthesized 16α-[^{125}I]iodo-dihydrotestosterone and shown that its binding affinity for the androgen receptor *in vitro* is only 0.01 (1%) of that of 5α-dihydrotestosterone.

However, the factors that govern the relative accumulation of steroids by target tissues *in vivo* are complex, involving not only the affinity of the hormone for the appropriate target tissue receptor protein, but also the dose of steroid administered, its metabolism and the hormonal status of the animal [13, 14]. In the case of the androgens in particular the main circulating hormone, testosterone, is first converted in most target tissues to 5α-dihydrotestosterone. Both testosterone and 5α-dihydrotestosterone bind to the androgen receptor but the former has only one tenth the affinity of the latter [15]. Nevertheless, from studies in the rat, we have previously demonstrated that a greater proportion of radioactive label is retained by the prostate after the injection of [^{3}H]testosterone than [^{3}H]5α-dihydrotestosterone. This result is due, at least in part, to the faster metabolism of 5α-dihydrotestosterone [16] and the return of non-receptor-binding metabolites to the general circulation (unpublished observations). We therefore considered that in spite of Hoyte's findings we would synthesize 16α-[^{125}I]iodotestosterone and evaluate its use for imaging androgen receptor positive tumours.

‡ Since the submission of this manuscript the Chemistry Department of Westfield College has moved to Queen Mary College, Mile End Road, London E1 and R. D. Farrant to the Wellcome Research Laboratories, Beckenham, Kent.

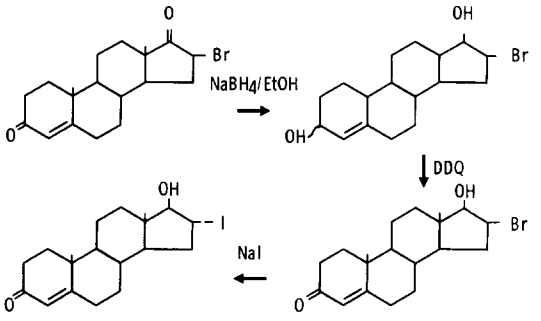

Fig. 1. Schematic diagram of the synthetic route from 16β-bromo-androstenedione to 16α-iodo-testosterone.

MATERIALS AND METHODS

Chemical synthesis

16α-Iodo-testosterone was synthesized from 16β-bromo-androstenedione (shown schematically in Fig. 1) which was in turn synthesized from dehydro*epi*androsterone by the method of Fajkos and Sorm [17].

16β-*Bromo--androstenedione to* 16β-*bromo-testosterone*

16β-Bromo-androstenedione (0.8 g) was dissolved in ethanol (256 ml) and cooled to 4°. Sodium borohydride (0.16 g) was added and the mixture incubated at 4° for 24 hr. At this stage silica gel GF254 thin layer chromatographic analysis in a mixture of toluene, ethanol and ethyl acetate (9:1:1, v/v/v) revealed one major and three minor products, none of which were u.v. absorbing. The reaction was stopped by the addition of water (300 ml). The products were extracted into ether which was dried over $MgSO_4$. The ether was taken to dryness on a rotary evaporator. No attempt was made to purify the product at this stage. The material was redissolved in dry 1,4 dioxan (24 ml) to which was added dichloro-dicyano-benzo-quinone (DDQ) (0.5 g), after 24 hr at room temperature more DDQ (1.0 g) was added and the mixture incubated for a further 24 hr.

Thin layer chromatography revealed one major (R_f 0.41) and two minor products, all u.v. absorbing. The reaction was stopped by the addition of ether (100 ml). The ethereal solution was washed with water, taken to dryness and fractionated by silica gel column chromatography, eluted with toluene and ethyl acetate (9:1, v/v). The major product was identified by running the fractions on a thin layer chromatogram. The appropriate fractions were combined and taken to dryness.

Recrystallization from methanol yielded a material melting at 189°, agreeing with the published melting point of 16β-bromo-testosterone. Final confirmation of the structure was established by ^{1}H NMR (Table 1).

16β-*Bromo-testosterone to* 16α-*iodo-testosterone*

This conversion was achieved by refluxing pure 16β-bromo-testosterone with sodium iodide (10-fold excess) in acetone. After 12 hr the product formed was purified by reverse phase high pressure liquid chromatography using a Waters Associates instrument fitted with a micro-bondapak C_{18}, 9.6 × 300 mm column. An isochratic elution system of 70% methanol in water was used at a flow rate of 3.5 ml/min. 16α-Iodo-testosterone (detected by u.v. absorption at 245 nm) was eluted after 18.25 min. The structure of the material was confirmed by ^{1}H

Table 1. ^{1}H NMR of (a) 16α-iodo-androstenedione 100 MHz, (b) 16α-iodo-testosterone 500 MHz, (c) 16β-bromo-androstenedione 100 MHz, (d) 16β-bromo-testosterone 100 MHz; in $CDCl_3$ at ambient temperature 22°

δ (ppm)	Assignment	δ (ppm)	Assignment
(a) 0.95	18 methyl	(c) 1.14	18 methyl
1.23	19 methyl	1.23	19 methyl
1.0–2.75	methylene envelope	0.84–2.82	methylene envelope
4.86	16β	4.11	16α
5.75	4	5.75	4
(b) 0.72	18 methyl		
0.9–1.22	14*, 9* and 12α*		
1.01	7α		
1.13	19 methyl		
1.37	8		
1.41	15α*	(d) 0.96	18 methyl
1.53	11β	1.21	19 methyl
1.56	Residual H_2O	0.71–2.75	methylene envelope
1.6–1.74	1α* and 11α*	3.37	17α
1.82	12β	4.61	16α
1.97	7β	5.73	4
2.02	17β OH		
2.10	15β		
2.12	1β*		
2.23	6α		
2.26–2.42	6β, 2α and 2β		
3.95	17α		
4.03	16β		
5.68	4		

* Tentative assignments.

NMR (Table 1). γ_{max}^{KBr}: 3100–3500 broad (O—H), 1650 with shoulder 1620 (—C=C—C=O) cm^{-1}. The compound decomposed on heating above 130°.

16β-Bromo-androstenedione to 16α-iodo-androstenedione

Halide exchange was carried out as described above and the iodinated material similarly purified. 16α-Iodo-androstenedione was eluted after 14.10 min on the HPLC described above. The compound was characterized by ^1H NMR (Table 1). γ_{max}^{KBr}: 1650 with shoulder 1610 (—C=C—C=O), 1740 (C=O) cm^{-1}. The compound decomposed on heating above 100°.

Radioactive iodination reactions using $Na^{125}I$

16α-[^{125}I]iodo-testosterone and 16α[^{125}I]iodo-androstenedione were prepared as described below.

One microCurie (5 μl) of an aqueous solution of $Na^{125}I$ (New England Nuclear, Boston, MA) was placed in a Teflon screw-top micro-reaction vial of total volume 100 μl. An aqueous solution of $Na_2S_2O_3$ (1 mM, 5 μl) and freshly distilled methyl cyanide (100 μl) were added and the mixture taken to dryness under a stream of N_2. A solution of the respective bromo compound (20 μg) in 2-butanone (2 μl) was added and the mixture incubated at 75° overnight. The reaction products were analysed by silica gel GF254 thin layer chromatography in chloroform and methanol (99:1, v/v). The starting material was identified by its u.v. absorption and the radioactive products by radiochromatogram scanning.

In both cases a single radioactive product was formed, that from 16β-bromo-testosterone running slightly slower and that from 16β-bromo-androstenedione slightly faster than the respective starting materials. The radioactive compounds were purified by thin layer chromatography, the appropriate area cut out and eluted with ethanol. The identity of the material was checked in each case by diluting a small amount of the radioactive products with pure 16α-iodo-testosterone or 16α-iodo-androstenedione. The mixtures were analysed by HPLC and in both cases the radioactive material was eluted as a single peak coincident with the authentic chemical compound.

Animal studies

Animals. Wistar rats bred in the Courtauld Institute and maintained on standard diet were used. Where appropriate animals were castrated by the scrotal route under ether anaesthesia. Test substances were dissolved in ethanol which was made 10% with respect to normal saline. The compounds were administered by injection into the penile vein while the animal was under light ether anaesthesia.

Tissue distribution studies. Animals were killed at various times after the administration of test substances, tissues were sampled and taken for determination of radioactivity by direct gamma counting.

Subcellular fractionation. All procedures were conducted at 0–4°. Prostatic tissue (2.0 g) was minced with scissors and briefly homogenized (4 sec) using a Silverson mixer emulsifier in 7.5 vol of TES buffer (10 mM TES pH 7.0) containing 0.5 mM mercaptoethanol and 0.25 M sucrose. The homogenate was centrifuged at 400 *g* to yield a crude nuclear pellet and supernatant. This supernatant was centrifuged at 100,000 *g* for 1 hr (cytosol). The crude nuclear pellet was resuspended in TES buffer containing 2.2 M sucrose and 0.5 mM $CaCl_2$ and centrifuged at 100,000 *g* for 1 hr to yield a purified nuclear pellet. The pellet was resuspended in TES buffer (2.0 ml).

Metabolic studies. Animals were killed by decapitation 0.5 hr and 2 hr after the injection of 16α-[^{125}I]iodo-testosterone. Blood samples were collected and prostatic tissue removed. Samples of plasma, the purified nuclei and the post nuclear supernatant were extracted into $CHCl_3$. The $CHCl_3$ extracts were analysed by silica gel thin layer chromatography developed in $CHCl_3$: methanol (99:1 v/v). The area of the plate corresponding to 16α-iodo-testosterone was identified by comparison with standard plates run simultaneously. Each plate was divided into 1 cm sections and each section taken for determination of radioactivity.

Binding studies. Samples (1.0 ml) of prostatic cytosol were applied to a column (1 × 20 cm) of Sephadex G-25F and eluted in a descending direction at the maximum flow rate. The column was eluted with TES buffer containing 0.6 M NaCl and 5 mM sodium EDTA, 0.3 ml fractions were collected. The void volume was determined using dextran 2000.

RESULTS

The characterization of 16β-bromo-androstenedione and 16β-bromo-testosterone was achieved by melting point (which in each case agreed with published values) and by ^1H NMR at 100 MHz. The characterization of 16α-iodo-andostenedione and 16α-iodo-testosterone was conducted by i.r. and ^1H NMR at 100 and 500 MHz. The assignment of protons is detailed in Table 1.

In the absence of any reliable literature data on the 16α-iodo-steroids for comparison it was decided that further unambiguous proof of stereochemistry should be obtained. This was done at 500 MHz using nuclear Overhauser effect difference spectra (NOEDS) measurement on 16α-iodo-testosterone. Two NOEDS were performed using the methods of Hall and Sanders [18, 19] and explained in detail by Sanders and Mersh [20] in the review of double resonance techniques.

In the first experiment irradiation of the 18 methyl resulted in enhancement to the signals of the 16β, 15β, 8β, 11β, and 12β, protons and in enhancement also of the 17β hydroxyl proton. This in turn produced saturation transfer effects to the residual water in the solution. In the second experiment, irradiation of the putative 16β proton resonance resulted in enhancements of the 15β and 18 methyl signals together with the same 17β hydroxyl and water saturation transfer effects as in the first case. Both results compliment each other and prove unequivocally the relative stereochemistry of the molecule to be the desired 16α-iodo-testosterone. The stereochemistry of 16α-iodo-androstenedione was confirmed by comparison.

The chemical identity of 16α-[^{125}I]iodo-testosterone and 16α-[^{125}I]iodo-androstenedione were established by chromatography of the radiolabelled

Table 2a. Distribution of 16α-[^{125}I]iodo-androstenedione (cpm/g tissue) between tissues of the rat after *in vivo* injection, 0.2 μCi i.v. to intact animals

Tissue	Time after injection				
	1 hr	2 hr	4 hr	6 hr	15 hr
Blood	78,266	31,447	13,466	13,583	595
Prostate	24,536	25,162	19,020	11,078	736
Brain	2590	2100	2000	2250	290
Kidney	37,023	25,071	49,560	19,640	629
Heart	21,054	11,215	5800	5576	468
Lung	6626	29,150	15,771	12,000	818
Spleen	26,353	19,963	11,700	9720	380
Liver	27,000	21,225	9709	10,666	690

material diluted with authentic unlabelled material. In each case a single radioactive spot was identified coincident with the respective unlabelled material.

16α-[^{125}I]iodo-testosterone or 16α-[^{125}I]iodo-androstenedione was injected into normal and 24 hr castrate male rats. The distribution of these compounds between tissues are detailed in Tables 2 and 3. No significant prostatic accumulation against the blood gradient could be detected with either compound. The results were very similar in both normal and 24 hr castrate animals.

Further experiments were conducted with 16α-[^{125}I]iodo-testosterone *in vivo* to examine its metabolism and subcellular distribution in the prostate. These experiments were carried out on animals which had been injected 0.5 hr or 2 hr previously. The analysis of metabolites by thin layer chromatography is shown in Fig. 2. In the plasma the parent compound was the major circulating species but a less polar metabolite, which increased with time, was also observed, running with the solvent front. In the prostate the cytosol also contained the parent compound and the material running with the solvent front and in addition a less polar metabolite running slightly faster than the 16α-iodo testosterone. The prostatic nuclei contained no parent compound. Some of the less polar material at the solvent front was detected but the major metabolite was a very polar compound running near the start line. Based on the known metabolism of testosterone by the prostate we postulate that the slightly less polar

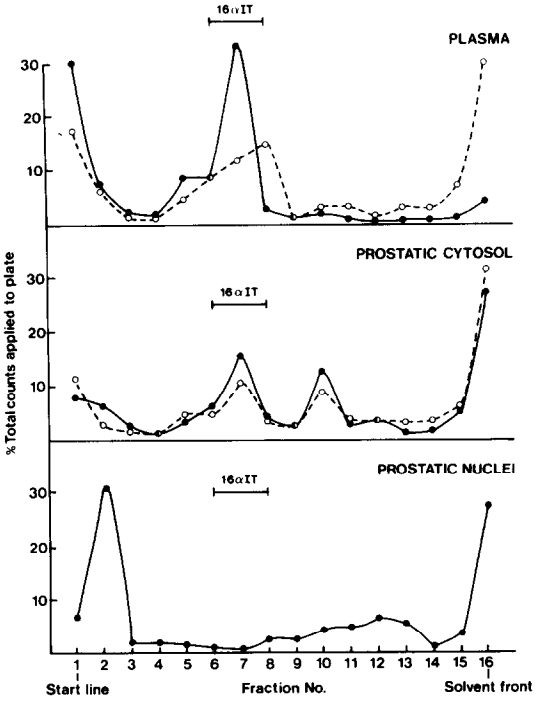

Fig. 2. Analysis by thin layer chromatography of metabolites extracted from (a) plasma, (b) prostatic cytosol, (c) prostatic nuclei after the injection 0.5 hr (●—●—●) and 2 hr (○--○--○) previously of 16α-[^{125}I]iodo-testosterone, 0.2 μCi i.v. to 24 hr castrate rats.

metabolite in the cytosol is 16α-iodo-dihydrotestosterone and that the more polar metabolite in the nuclei is a diol.

The distribution of radioactivity between the crude nuclear pellet and post nuclear supernatant are shown in Table 4. For comparison data from similar experiments in which animals had received [^{3}H]testosterone are included. Very little of the radioactivity after injection of 16α-[^{125}I]iodo-testosterone can be detected in the crude nuclear pellet after 0.5 hr and it is also very labile, by 2 hr post injection most of this radioactivity had been lost.

The distribution of counts between protein bound and free forms was determined in the prostatic cyto-

Table 2b. Distribution of 16α-[^{125}I]iodo-androstenedione (cpm/g tissue) between tissues of the rat after *in vivo* injection, 0.2 μCi i.v. to 24 hr castrate animals

Tissue	Time after injection							
	15 sec	30 sec	60 sec	2 hr	4 hr	6 hr	8 hr	15.5 hr
Blood	59,202	39,246	31,206	19,164	22,788	16,139	14,904	1159
Prostate	35,832	12,192	22,896	18,210	18,738	17,650	17,675	900
Brain	5550	4578	1920	1518	2745	2494	1583	379
Kidney	52,158	35,262	25,800	12,642	17,298	10,938	13,047	1338
Heart	19,152	13,248	11,100	5610	6683	5230	—	592
Lung	20,064	46,423	42,300	21,096	7255	19,028	17,944	1132
Spleen	5016	18,588	18,780	11,028	11,836	8589	6830	897
Liver	45,690	18,284	15,438	9900	10,561	9000	9750	1000

Table 3a. Distribution of 16α-[^{125}I]iodo-testosterone (cpm/g tissue) between tissues of the rat after *in vivo* injection, 0.2 μCi i.v. to intact animals

Tissue	Time after injection				
	0.5 hr	2 hr	4 hr	7 hr	24 hr
Blood	41,982	15,600	17,277	14,321	2420
Prostate	25,489	18,925	11,400	8612	970
Brain	28,325	2917	1945	1292	724
Kidney	65,972	20,997	18,400	11,080	2345
Heart	33,761	473	6115	4849	834
Lung	44,608	15,685	11,297	14,303	2203
Spleen	31,706	9193	12,363	7843	1356
Liver	272,000	56,828	23,674	14,261	3938

sol 0.5 hr after injection of the compound. Almost all the radioactivity was included in the gel, corresponding to non-protein bound material. In contrast after the injection of [^{3}H]testosterone, 25% or more of the radioactivity was collected in the void volume, indicating substantial binding to protein (Fig. 3).

DISCUSSION

Physiological doses of testosterone are accumulated in the prostate against the blood gradient, however, after insertion of the iodine atom at position 16α in the testosterone molecule no selective prostatic accumulation can be detected.

Table 4. Ratio of radioactivity in the crude nuclei and cytosol of the prostate 0.5 hr and 2 hr after the injection of 16α[^{125}I]iodo-testosterone 0.2 μCi or [^{3}H]testosterone 10 μCi i.v. to 24 hr castrate male rats

Injected material	Nuclear/cytosol ratio	
	0.5 hr	2 hr
16α[^{125}I]testosterone	0.25–0.33	0.1
[^{3}H]testosterone	1.82 ± 0.2	1.5 ± 0.3

The mechanisms underlying prostatic retention of testosterone involve the stability of this steroid in plasma and its binding to protein in the target tissue either before or after metabolic conversion to 5α-dihydrotestosterone. Studies with 16α-[^{125}I]iodo-testosterone indicate that a large proportion of the injected compound circulates unchanged in the plasma. It is, however, metabolized by the prostate. We speculate that a major prostatic metabolite is 16α-iodo-5α-dihydrotestosterone and therefore that 16α-iodo-testosterone is a substrate for the 5α-reductase enzyme.

Table 3b. Distribution of 16α-[^{125}I]iodo-testosterone (cpm/g tissue) between tissues of the rat after *in vivo* injection, 0.2 μCi i.v. to 24 hr castrate animals

Tissue	Time after injection						
	0.5 hr	1 hr	2 hr	4 hr	7 hr	12 hr	24 hr
Blood	46,506	47,634	42,237	43,295	38,400	17,414	3400
Prostate	53,733	35,700	42,031	46,400	27,400	30,700	2435
Brain	50,544	14,935	7080	3378	2821	2105	702
Kidney	99,264	53,119	43,971	33,646	32,966	18,678	2998
Heart	61,020	24,450	10,940	16,566	12,100	9161	1581
Lung	62,400	43,824	40,680	43,560	27,128	14,233	3032
Spleen	40,753	26,347	22,330	25,656	12,620	21,004	1885
Liver	304,819	97,323	60,848	41,981	26,748	13,233	4237

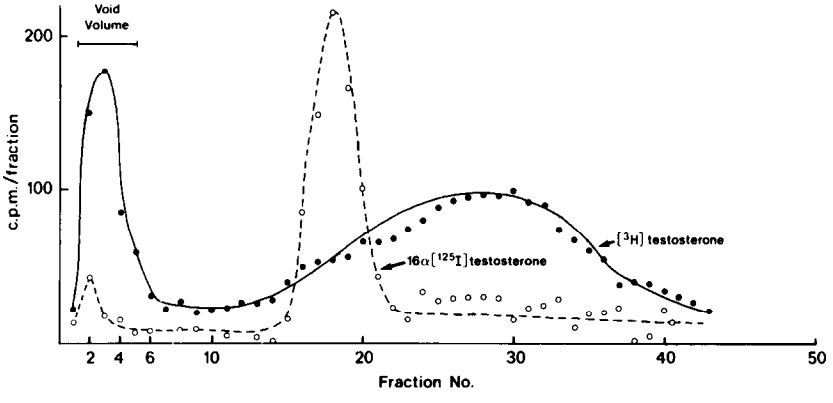

Fig. 3. Sephadex G-25F chromatography of prostatic cytosol 0.5 hr after the *in vivo* injection of 16α-[^{125}I]iodo-testosterone 0.2 μCi (○--○--○) or [^{3}H]testosterone 10 μCi (●—●—●) to 24 hr castrate rats.

Subcellular distribution studies demonstrate that neither 16α-[^{125}I]iodo-testosterone nor its metabolites are protein bound in the soluble fraction and neither are they substantially translocated to or bound in the nucleus. These observations confirm those of Hoyte *et al.* [12] that the 16α substitution reduces the binding affinity for the androgen receptor. It is unfortunate that the factors which cause the testosterone to be accumulated better than dihydrotestosterone *in vivo* do not extend sufficiently to the iodo compounds. It is, nevertheless, interesting that the substitution of an iodine atom at position 16α should dramatically affect the binding affinity of androgens to their receptor, whereas a similar substitution in oestradiol does not alter its binding affinity for the oestrogen receptor. We must thus, conclude that further attempts to develop radiopharmaceuticals for imaging androgen receptor positive tissue must concentrate on substitution of iodine at positions other than 16α in the steroid nucleus.

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