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N, *N*-Diethylfluoromethyltamoxifen: synthesis, assignment of ^{1}H and ^{13}C spectra and receptor assay

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Summary — *(cis)* and *(trans)N,N*-diethylfluoromethyltamoxifen (1-[4-(2-diethylaminoethoxy)phenyl]-1,2-diphenyl-5-fluoro-1-pentene) were synthesized from clomiphene in a three-step procedure and both isomers were differentiated by the concerted application of two-dimensional NMR techniques. In *in vitro* estrogen receptor assay from pig uterus using [^{3}H]-estradiol (5 nM), the IC_{50} values were: *N,N*-diethylfluorotamoxifen *cis* 5 μM, *trans* 1 μM; tamoxifen 30 μM. In human MCF7 cell growth assay, the IC_{50} values were *cis* 4.5 μM, *trans* 11.8 μM, tamoxifen 11.0 μM. The data suggest that the fluoro analog of tamoxifen has potential for imaging estrogen receptors by positron emission tomography (PET) and may be used to predict the efficacy of breast tumor therapy.

fluorotamoxifen / synthesis / receptor assay / 2-D NMR

Introduction

Tamoxifen (TX) (1-[4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenyl-1-butene) has been widely used in the treatment of human breast tumors. It is believed that this compound binds to cytoplasmic estrogen receptors and is translocated to cell nuclei, where it prevents cell proliferation [1].

This study is aimed at developing agents for imaging estrogen receptors using positron emission tomography (PET). Aliphatic fluorination of TX was chosen based on relative simplicity of its synthetic route and expected high specific radioactivity [2]. In the preparation of *N,N*-diethylfluorotamoxifen, elimination of a tosyl group producing a butadiene derivative was observed [3]. Increasing the length of the side chain by one carbon resulted in the synthesis of *N,N*-diethylfluoromethyl-tamoxifen from the corresponding tosylmethyl derivative with no elimination. Tamoxifen has both *cis* and *trans* isomers. The *trans* isomer has been used for breast tumor treatment. In this study, we described 2-dimensional NMR techniques to differentiate *cis* (*E*) and *trans* (*Z*) isomers. Here, we also report the synthesis and the *in vitro* estrogen receptor binding assay and MCF7 cell inhibition studies for the fluoro analog of tamoxifen.

Materials and methods

Chemistry

1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-1-penten-5-ol (N,N-diethylhydroxymethyltamoxifen)

Clomiphene (*E/Z*; 55/45; Sigma, St Louis, MO) (3.8 g, 9.3 mmol) was dissolved in THF, cooled to –40°C and treated with *t*-BuLi (1 M in pentane, 20 mmol). After 10 min, oxetane (6 ml, 93 mmol) was added and the mixture was stirred for an additional 4 h at room temperature and poured into water (20 ml). The product was extracted with ether and chromatographed on a silica gel column using ether/petroleum ether/triethylamine (5:5:1) as eluant to yield *Z* isomer (1 g, 25%, mp 94°C); R_f = 0.3 and *E* isomer; (1 g, 25%, mp 86°C); R_f = 0.2 (silica gel plate, Et_2O/ petroleum ether/Et_3N, 5:5:1). *Z* ^{1}H-NMR δ 1.02 (t, *J* = 7.0 Hz, 6, CH_3CH_2N), 1.59 (Pent, *J* = 7.3 Hz, 2, $CH_2CH_2CH_2OH$), 1.65 (br S, 1, OH), 2.45–2.60 (m, 6, OCH_2CH_2N and CH_3CH_2N), 2.77 (t, *J* = 6.4 Hz, 2, $CH_2CH_2CH_2OH$), 3.50 (t, *J* = 6.5 Hz, 2, $CH_2CH_2CH_2OH$), 3.90 (t, *J* = 6.4 Hz, 2, OCH_2CH_2N), 6.53 (d, *J* = 8.4 Hz, 2, ArH 2,6 to OCH_2) 6.75 (d, *J* = 8.4 Hz, 2, ArH 3,5 to OCH_2), 7.11–7.35 (m, 10, ArH); *m/z* 429 (35, M^+). Anal $C_{29}H_{35}NO_2$ (C, H, N). Calc, C: 81.08, H: 8.21, N: 3.26; found, C: 80.56, H: 7.94, N: 3.32. *E* ^{1}H-NMR δ 1.02 (t, *J* = 6.6 Hz, 6, CH_3CH_2N), 1.25 (br S, 1, OH), 2.48–2.64 (m, 8, CH_3CH_2N, OCH_2CH_2N and $CH_2CH_2CH_2OH$), 2.84 (t, *J* = 6.2 Hz, 2, $CH_2CH_2CH_2OH$), 3.52 (t, *J* = 6.2 Hz, 2, $CH_2CH_2CH_2OH$), 3.99 (t, *J* = 6.3 Hz, 2, OCH_2CH_2N), 6.85 (d, *J* = 8.1 Hz, 2, ArH 2,6 to OCH_2),

7.16–7.51 (m, 10, ArH), 7.97 (d, J = 7.6 Hz, 2, ArH 3,5 to OCH_2); m/z 429 (30, M^+); m/z 429 (30, M^+), Anal $C_{29}H_{35}NO_2$ (C, H, N).

1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-5-tosyl-1-pentene(N,N-diethyltosylmethyltamoxifen)
Hydroxymethyltamoxifen (0.5 g, 1.17 mmol) was dissolved in CH_2Cl_2 and cooled to 0°C. Pyridine (0.6 ml) and tosyl chloride (266 mg, 1.4 mmol) were added. The crude mixture was stirred for 2 h and then washed with water, evaporated to dryness and chromatographed on a silica gel column using ether/petroleum ether/triethylamine as eluant to yield the Z (200 mg, 26%); R_f = 0.35 and E analogs (180 mg, 20%), R_f = 0.60 (silica gel plate, Et_2O/petroleum ether/Et_3N; 5:5:1), m/z 583 (10, M^+). Anal $C_{36}H_{41}NO_4S{\cdot}0.5H_2O$ (C, H, N).

1-[4-(2-Diethylaminoethoxy)phenyl]-1,2-diphenyl-5-fluoro-1-pentene (N N-diethylfluoromethyltamoxifen)
The tosyl analog of tamoxifen (117 mg, 0.2 mmol) was dissolved in THF (0.4 ml). Tetrabutylammonium fluoride (0.5 ml, 1 M in THF) was added and the reaction heated to 80°C for 30 min. The mixture was then hydrolyzed with 6 N HCl (0.2 ml) for 10 min, and the product chromatographed on a silica gel column using the same eluant as above to yield the E isomer 52 mg (60%), R_f = 0.60 or Z isomer (40 mg, 46%), R_f = 0.80 (silica gel plates, Et_2O/petroleum ether/Et_3N; 5:5:1), m/z 431 (40, M^+). Anal $C_{29}H_{34}NOF$ (C, H, N).

Pharmacology

In vitro estrogen receptor assay

The test compounds affinity for binding to the estrogen receptor was determined with a modification of the procedure reported by others [4, 5]. Briefly, uteri (30 g) obtained from a domestic pig (30 kg) were homogenized in Tris buffer (10 mM, pH 7.4), (80 ml), which contained EDTA (1.5 nM) and sodium azide (3 nM). The homogenate was centrifuged at 100 000 g for 1 h at 4°C. The cytosol fraction was then pretreated with dextran-coated charcoal as described [4]. To investigate the nature of estradiol's interaction with the estrogen receptor site, a saturation curve was obtained for [^{3}H]-estradiol (10 μM to 0.1 nM) in either the presence or absence of excess estradiol (10 μM). Uterine cytosol was incubated at 4°C for 2 h with [^{3}H]-estradiol (5 nM/tube) and competitor (ranging from 1 mM to 10 nM) or with estradiol (10 μM) (nonspecific). The concentration of test compounds that decreased specific radioligand binding by 50% (IC_{50}) was determined. Protein concentrations were determined according to the method of Lowry *et al* [6].

MCF7 cell growth assay

The human breast tumor MCF7 cell line was cultured in MEM (Eagles) media in a 5% CO_2 atmosphere with 10% fetal calf serum. The medium was supplemented with 1 mM sodium pyruvate and 100 μm of nonessential amino acids. The cell line was screened routinely for mycoplasma contamination using the GenProbe kit (Fisher Scientific, Houston, TX). Cells were trypsinized and plated at a density of 5 000 cells/well in 96-well microtiter plates and allowed to attach and recover for 24 h. The medium was removed by aspiration and replaced with filter sterilized drug (concentration 0.1 mM–10 nM) in fresh medium. The cells were incubated for 72 h and then stained by using the MTT tetrazolium dye assay according to Mosmann [7]. After the medium was removed, the blue formazan product was solubilized in 50 μl DMSO/well. Plates were shaken for 1 min and read on a Dynatech MR600 microplate reader within 2 h at a transmission wavelength of 570 nm and reference wavelength of 630 nm.

Results and discussion

^{1}H-NMR assignment

The assignment was carried out by 2-dimensional NMR techniques including COSY, long range COSY HC COSY and long-range HC COSY (COSY: homonuclear chemical shift correlation; HCCOSY: heteronuclear chemical shift correlation). The aromatic portion is subdivided into three isolated spin systems at 200 MHz. In the Z isomer, two spin systems were readily established for aromatic protons a and b [8, 9]. A correlation among the H_1 methylene protons (resonate at 2.76 ppm for E (fig 3) and 2.55 ppm for Z (fig 4)), the H_2 geminal methylene protons (resonate at 1.79 ppm for E and 1.80 ppm for Z), and H_3 protons (resonate at 4.38 ppm for *cis* and 4.42 ppm for *trans*) was observed during the analysis of the COSY spectrum as shown in table I and 2-D-NMR spectra. In addition, the protons at the 4- and 5-ethylene bridge correlated with each other according to COSY spectrum analysis. H_4 resonated downfield at 3.99 ppm (E) and 3.91 ppm (Z), whereas H_5 resonated at 2.8 ppm (E) and 2.79 ppm (Z). H_6 protons of the ethyl group showed a quadruplet (resonate at 2.57 ppm for E and 2.57 ppm for Z), which directly correlates with H_7 methyl protons at 1.01 ppm (E) and 1.03 ppm (Z).

^{13}C-NMR assignment

Proton resonance assignments were unequivocally assigned by a COSY spectrum. Protonated carbon resonance was assigned from a HC-COSY spectrum. The chemical shift for E and Z isomers is shown in table I.

Table I. Proton (J_{HH}) and carbon (J_{CF}) resonance shift assignments for *N,N*-diethylfluoromethyltamoxifen.

Proton (± 0.02 ppm)		*No of protons*	*Multiplicity J_{HH} (Hz)*		*No of carbons*	*Carbon J_{CF} (Hz)*	
trans	*cis*	*cis/trans*	*trans*	*cis*		*trans*	*cis*
7.25 Har	7.23	10H	m	m	6C	Car	Car
						130–157	130–157
					10C	Car	Car
						126–132	126–131
6.79 Hb	7.10	2H	d	m	1C	Cb	Cb
			(6.8)			113.5	114.2
6.56 Ha	7.00	2H	d	m	1C	Ca	Ca
			(6.8)			113.5	114.2
4.42 H_3	4.38	2H	dt	dt	1C	C_3	C_3
			(47.3)	(47.3)		85.2	83.5
			(6.1)	(6.10)		(d; 165)	(d; 165)
3.91 H_4	3.99	2H	t	t	1C	C_4	C_4
			(6.4)	(6.37)		66.3	66.6
2.79 H_5	2.80	2H	t	t	1C	C_5	C_5
			(6.4)	(6.37)		51.7	51.9
2.56 H_6	2.57	4H	m	m	2C	C_6	C_6
						47.8	47.9
2.55 H_1	2.76	2H	m	m	1C	C_1	C_1
						31.6	31.5
						(d; 5.5)	(d; 5.5)
1.8 H_2	1.79	2H	m	m	1C	C_2	C_2
						29.8	29.9
						(d; 19.5)	(d; 19.5)
1.03 H_7	1.01	6H	t	t	2C	C_7	C_7
			(7.2)	(7.2)		11.8	11.8

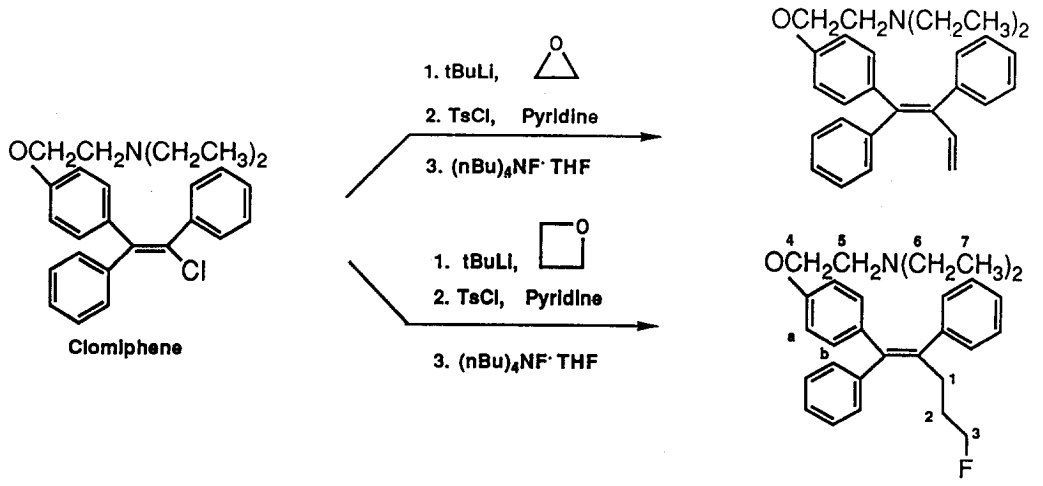

Fig 1. Synthesis of fluoromethyl analogs of tamoxifen.

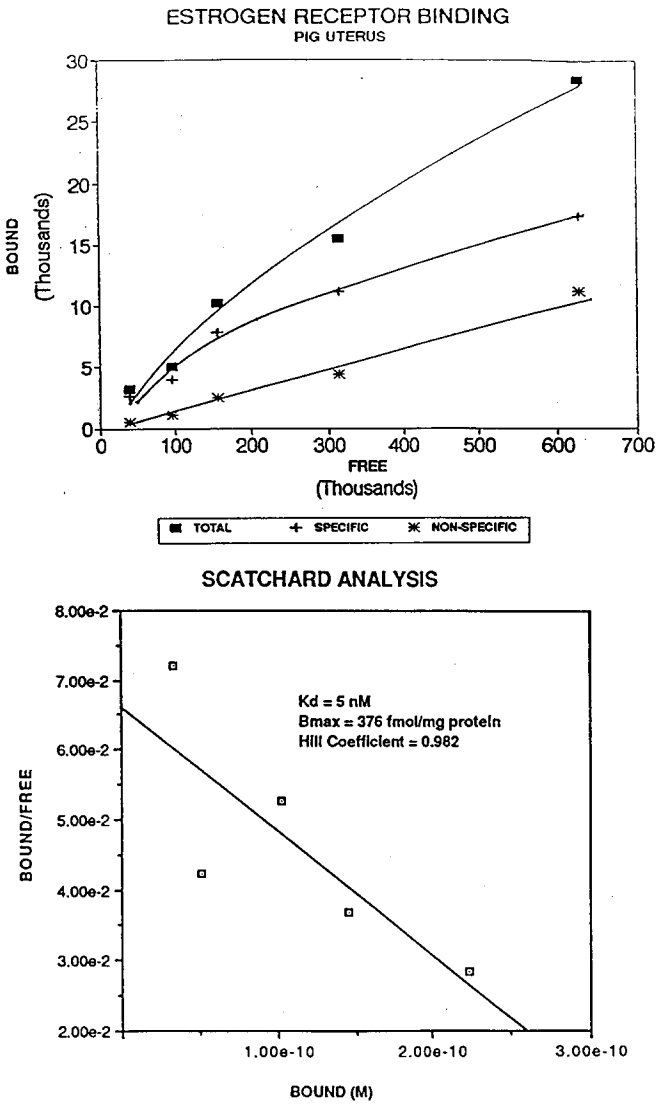

Fig 2. *In vitro* saturation experiment and Scatchard plot for estrogen receptor assay.

Receptor assay

For [^{3}H]-estradiol binding, Hill analysis (0.995) indicated that estradiol has competitive reversible binding. Scatchard analysis indicated a single class of binding sites for estradiol (fig 2). The IC_{50} of *N,N*-diethyl-fluoro analogs of tamoxifen is shown in table II. Both analogs bind to estrogen receptor to a greater degree than tamoxifen, the *Z* isomer having a greater affinity for the receptor than the *E* isomer. Since the fluorine atom is close to the hydrogen atom in size difference, the binding affinity between the fluoro analog and tamoxifen may be due to an all inductive effect produced by the halogen. The *trans* isomer apparently fits the estrogen receptor better than the *E* isomer. The concentration of test compounds required to inhibit 50% of MCF7 cell growth was determined, and the results are summarized in table III. Unlike observation made in estrogen receptor binding, the *E* isomer was more potent than the corresponding *Z* isomer. It has been reported that in non-halogenated analogs of tamoxifen, an *E* isomer could partially be converted to the more active *Z* isomer during cell culture experiments, as reported by McCague and Leclercq [10] and Katzenellenbogen *et al* [11]. However, this *trans/cis* interconversion will only take place in 4-hydroxylated derivatives. Our compound has an extra CH_2 and a halogen on the side chain which is different from non-halogenated tamoxifen. Our data indicate that the isomer is more lethal than the *Z* isomer. This lethal effect could be due to the intramolecular hydrogen

Table II. Effect of tamoxifen analogs on estrogen receptor binding in pig uterus.

Compound	IC_{50} (μM) ± *SD*[a]	*RBA*[b]
Tamoxifen (*Z*)	30 ± 1.21	
N-N-Diethylfluoromethyltamoxifen (*E*)	5 ± 0.3	6
(*Z*)	1 ± 0.4	30

[a]Data represent the average of three experiments; in each experiment data were reproduced in triplicate. [b]Relative binding affinity (RBA) is the ratio between the concentration of tamoxifen and competitor (x 100%) required to decrease the amount of bound [^{3}H]-estradiol by 50%.

Table III. Effects of tamoxifen analogs on human breast tumor cell growth[a].

Compound	IC_{50} (μM) ± *SD*[b]
Tamoxifen (*Z*)	11.0 ± 0.21
N-N-Diethylfluoromethyltamoxifen (*E*)	4.5 ± 0.04
(*Z*)	11.8 ± 0.40

[a]Data represent the average of three experiments; in each experiment data were reproduced in triplicate. [b]IC_{50} indicates the concentrations of test compounds required to inhibit 50% of MCF7 cells growth. Data represent the mean of three studies of each compound.

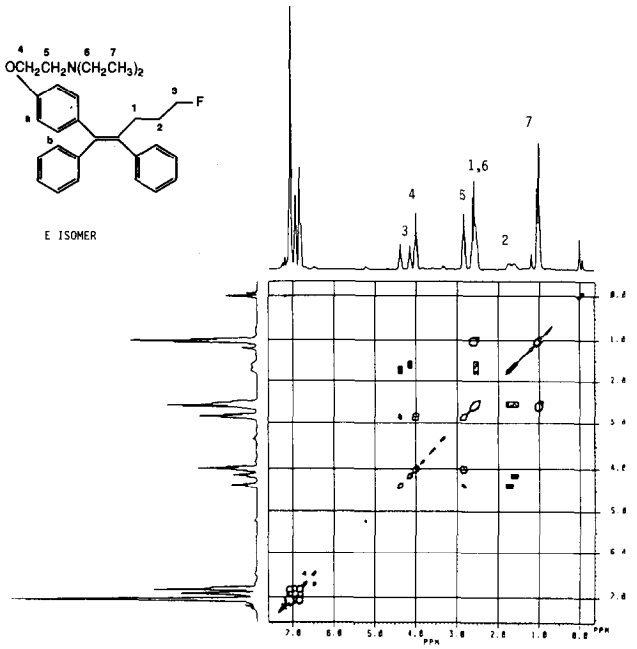

Fig 3. COSY spectrum of the *E*-fluoro analog of tamoxifen. The spectra were taken as 256 x 1 K complex points and were processed using sinusoidal multiplication. Both Fourier transforms and zero filling afforded final data matrices consisting of 512 x 512 real points; all points were symmetrized prior to plotting. Proton identities are labeled on the high resolution reference spectrum plotted beneath the contour plot.

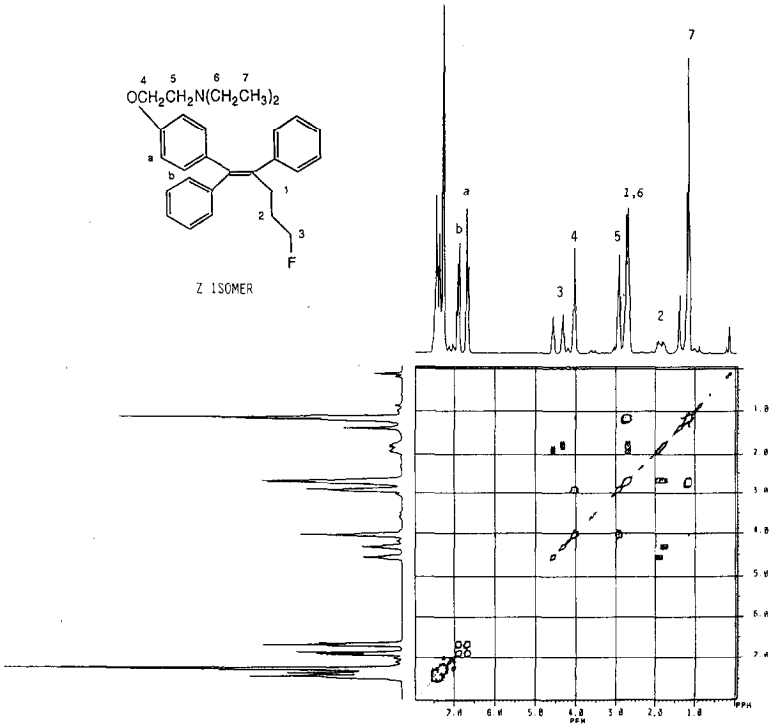

Fig 4. COSY spectrum of the *Z*-fluoro analog of tamoxifen. The spectra were taken as 256 x 1 K complex points and were processed using sinusoidal multiplication. Both Fourier transforms and zero filling afforded final data matrices consisting of 512 x 512 real points; all points were symmetrized prior to plotting. Proton identities are labeled on the high resolution reference spectrum plotted beneath the contour plot.

bonding caused by the *E* isomer. This hydrogen bonding effect could facilitate the molecule to chelate to the tumor cell phospholipid membrane and this change in membrane permeability could cause cell death. In summary, the fluoro analog of tamoxifen binds to estrogen receptor with a 30-fold greater affinity than tamoxifen and can be prepared by a three-step synthetic procedure (fig 1). Such a procedure is also suitable for preparing a compound of high specific radioactivity. The data reflect the potential usefulness of applying the fluoro analog of tamoxifen to image estrogen receptors by PET.

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