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EVIDENCE THAT TAMOXIFEN BINDS TO CALMODULIN IN A CONFORMATION DIFFERENT TO THAT WHEN BINDING TO ESTROGEN RECEPTORS, THROUGH STRUCTURE–ACTIVITY STUDY ON RING-FUSED ANALOGUES

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Abstract—A ring-fused analogue of tamoxifen, which had previously been shown to have practically identical estrogen receptor (ER) affinity and antitumour potency against estrogen responsive cells as tamoxifen, failed to inhibit calmodulin-dependent cyclic AMP phosphodiesterase. The substitution of an extra methyl group into the ring-fused analogue, at a position which the ethyl group of tamoxifen can occupy in one of its conformations, restored the calmodulin inhibition. Also, the replacement of the tamoxifen ethyl group by methyl diminishes calmodulin inhibition. Direct interaction of these tamoxifen analogues with calmodulin was demonstrated through the use of the fluorescent probe, 2-*p*-toluidinyl-naphthalene-6-sulfonic acid (TNS). These findings lead to the conclusion that tamoxifen binds to calmodulin in a conformation not accessible to the fused analogue and therefore likely to be different to that which binds to the ER. Also, the results on the ring-fused analogues indicate that the calmodulin binding cannot be essential for antitumour activity.

Key words: antiestrogens; triphenylethylenes; cAMP phosphodiesterase; benzocycloheptenes

Since the discovery that tamoxifen is an antagonist of the calmodulin activation of cAMP‡ phosphodiesterase [1] in addition to its action on ER, calmodulin inhibition has been put forward as a possibly important mechanism by which the triarylethylene antiestrogens exert their antitumour action [2–4]. This theory has been especially supported by more recent observations that calmodulin mediates phosphorylation of ER which is necessary for activation of this receptor [5] and that calmodulin binds directly to ER [6] and tamoxifen inhibits this binding [7].

In order to gain further insight into such issues we have been examining a variety of analogues of tamoxifen with respect to the profile of inhibition of calmodulin-dependent cAMP phosphodiesterase against antitumour potency. A particularly interesting series were those with a 4-substituent where we found a correlation between calmodulin inhibition and cytotoxic action against a hormone-dependent cell line [8]. The pyrrolidino-4-iodotamoxifen analogue (idoxifene) was the most potent calmodulin antagonist as well as having a greater binding affinity for the ER compared to tamoxifen. Owing to its improved efficacy in animal studies [9], idoxifene is currently in Phase I/II clinical trials. In this present study, we describe our results with seven-membered ring-fused analogues of tamoxifen; the analogue **3** has now been extensively studied as an antiestrogen having practically identical activity against ER and against estrogen-dependent breast cancer cell lines as tamoxifen, but without the complications caused by potential isomerization of metabolites [10, 11]. In addition to testing the compounds against the calmodulin-dependent cAMP phosphodiesterase, the analogues were examined for a direct interaction with calmodulin by utilising the fluorescent probe, TNS. Previous workers have characterised the binding of TNS to calmodulin [12, 13] and used it to identify calmodulin antagonists [14, 15]. Following an initial unexpected result when it came to measuring the calmodulin inhibition by **3**, other compounds were prepared to support a theory of explanation and we can now identify conformational requirements for calmodulin inhibition. The structures of triarylethylene compounds are shown in Fig. 1.

MATERIALS AND METHODS

Materials. Pure pig brain calmodulin, cAMP and calmodulin deficient phosphodiesterase EC 3.1.4.17 (from beef heart) were obtained from Boehringer Mannheim (Lewes, U.K.). [8-^{3}H]cAMP (21.2 Ci/mmol) was purchased from Amersham International

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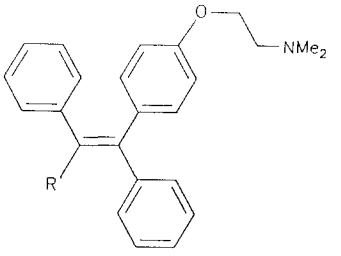
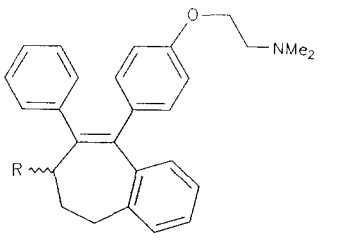
‡ Abbreviations: ER, estrogen receptor; THF, tetrahydrofuran; TNS, 2-*p*-toluidinyl-naphthalene-6-sulfonic acid; cAMP, cyclic adenosine monophosphate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride.



1, R = Et, tamoxifen
2, R = Me



3, R = H
4, R = Me

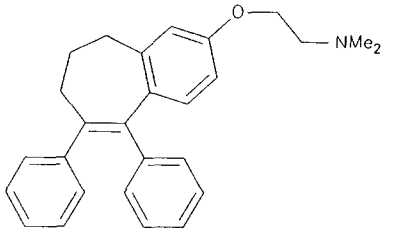


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Fig. 1. Structures of triarylethylene compounds.

(Bucks, U.K.). Dowex 1-x8 (200–400 mesh) anion exchange resin, snake venom (*Ophiophagus hannah*), W-7 and TNS, potassium salt were obtained from Sigma Chemical Co. (Poole, U.K.). Calmodulin (bovine brain) for the fluorescence assays was purchased from Calbiochem Novabiochem Ltd (Nottingham, U.K.). All other chemicals were of analytical grade.

Synthesis of tamoxifen analogues. Tamoxifen **1** and the ring-fused analogues **3** and **5** were synthesised by published methods [10, 16]. For the *C*-desmethyl analogue **2** *trans*-(E)-1-[4-(2-dimethylamino-ethoxy)phenyl-1,2-diphenyl-2-bromoethene [17] (81.4 mg, 0.19 mmol) was treated with methylzinc chloride (0.48 mmol; prepared from methyl lithium and zinc chloride in THF, 1 mL) under catalysis by chlorobenzylbis(triphenylphosphine)palladium (0) at reflux for 1 hr. After chromatography of the crude produce on silica, compound **2** obtained (20.9 mg) was isomerically pure by ^{1}H NMR spectroscopy. The synthesis of **4** was lengthy (Fig. 2) along the lines of that used to prepare **3** but requiring 3-methyl-1-benzosuberone prepared as follows: 1-benzosuberone was converted into its *O*-trimethylsilyl enol ether by treatment with chlorotrimethylsilane, triethylamine, and sodium iodide (each 1.2 equiv) in acetonitrile, and this enol ether (2.4 g, 10.34 mmol) was dissolved in THF (15 mL) treated with phenylselenyl bromide at −78°[prepared from diphenyl diselenide (3.23 g, 10.3 mmol) and bromine (1.65 g, 10.3 mmol)]. The resulting selenide (2.0 g) was recovered as a pale yellow oil after silica column chromatography and was dissolved in THF (150 mL) containing pyridine (2 mL) and oxidised with 30% hydrogen peroxide (3.5 mL) over 1 hr at ambient temperature. Recovery and silica chromatography gave 2,3-dehydro-1-benzosuberone (577 mg, 35%). A solution of this enone in THF (2 mL) was treated with lithium dimethylcuprate prepared from 1.25 equiv copper (I)bromide, dimethyl sulfide and 2.5 equiv, methyl lithium solution. Recovery and chromatography gave 3-methyl-1-benzosuberone (492 mg, 80%). A solution of (2-chloroethoxy)-4-bromobenzene (946 mg, 4.0 mmol) in THF (5 mL) at −78° was treated with *n*-butyllithium solution (3.75 mmol) and to this was added the methylbenzosuberone (467 mg, 2.68 mmol) and the mixture allowed to attain ambient temperature. Product recovery and chromatography on silica gave 7-methyl-9-[4-(2-chloroethoxy)]-6,7-dihydro-5*H*-benzocycloheptene (627 mg, 75%). NMR analysis showed it to contain 25% of the compound lacking the methyl group. Bromination with pyridinium hydrotribromide (1.2 equiv) in dichloromethane gave the 9-bromo derivative. Crystallisation from petrol removed much of the unmethylated contaminant. Treatment with phenylzinc chloride under palladium complex catalysis (method as previously described [10]) gave crystalline 8-phenyl derivative (92% yield). Finally, the dimethylamino function was introduced by treatment with dimethylamino in ethanol under pressure at 100° over 4 hr. After chromatography and recrystallisation from petrol, **4** was obtained (98.0 mg).

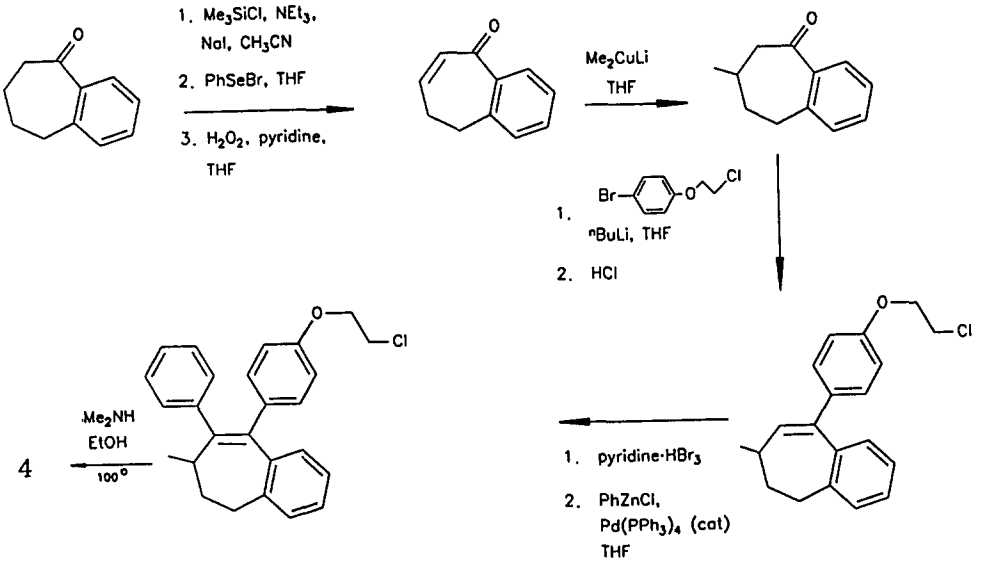

Fig. 2. Synthetic route of compound **4**.

Table 1. Inhibition of calmodulin-dependent cAMP phosphodiesterase activity and relative binding affinities (RBA) to the estrogen receptor for analogues of tamoxifen

Compound	IC_{50} value* (μM)	K_i value† (μM)	RBA (Estradiol = 100)
1 (*trans* tamoxifen)	6.75 ± 1.1	0.83	1.0‡
cis tamoxifen	6.50 ± 0.5	ND	0.1‡
Idoxifene	1.45 ± 0.1	0.33	4.0
2	19.00 ± 2.0	ND	0.1
3	>50	ND	2.7
4	8.40 ± 1.8	1.14	3.5
5	6.90 ± 1.4	0.98	0.1‡

ND = Not determined.

* IC_{50} values are the mean ± SE of triplicate determinations.

† The dissociation constants (K_i values) were derived from the double reciprocal plots, see Fig. 4.

‡ RBA values as reported in Ref. 20.

Assay of calmodulin-dependent phosphodiesterase. The enzyme was assayed using [8-^{3}H]cAMP as substrate [8, 18]. The tritiated AMP formed during the incubation was converted into tritiated adenosine by the 5′-nucleotidase in snake venom. Product nucleosides were separated from unreacted substrate using batch elution with Dowex anion exchange resin in 3 mM acetic acid. The tritiated adenosine was determined by liquid scintillation counting. The basal activity of cAMP phosphodiesterase (calmodulin independent) was determined by adding 1 mM EGTA to the assay medium. The incubation time and enzyme concentration was adjusted so that the substrate hydrolysis did not exceed 25% and the product formation was linear with time. The assays were carried out in the presence and absence of different concentrations of the compounds dissolved in dimethyl sulphoxide to give a final concentration of 1.5% in the assay mixture. This concentration of dimethyl sulphoxide had no significant effect on the calmodulin-dependent and -independent activity of the enzyme. The results are expressed as the concentration of inhibitor giving 50% inhibition of the calmodulin-dependent cAMP phosphodiesterase (IC_{50}). Analysis of the kinetic experiments was carried out using the Enzpack 3 (Biosoft) program in order to determine the K_i values and the mode of inhibition.

Fluorescence measurements. Fluorescence measurements were carried out at room temperature using a Perkin Elmer MPF-3L spectrophotometer. Samples were prepared in a total volume of 3 mL and consisted of 10 mM Tris–HCl buffer, pH 7.4, 0.5 mM $CaCl_2$, 2 μM calmodulin and 20 μM TNS. The triarylethylenes were dissolved in dimethyl sulphoxide and added to the samples to give a final

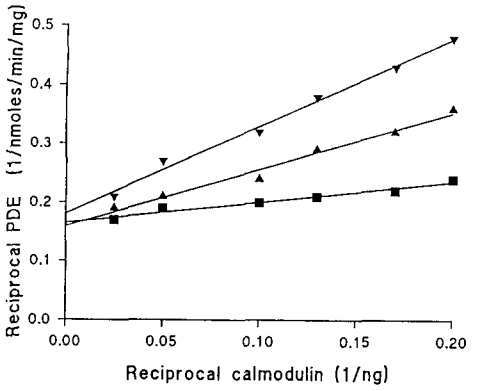

Fig. 3. Double reciprocal plot of the inhibition of calmodulin-stimulated phosphodiesterase (PDE) activity by compound **4** at 2 μM (▲) and 3 μM (▼). Control values (■) contained 1.5% dimethyl sulphoxide. Each point represents the mean (±10%) of triplicate determinations.

Table 2. Relative fluorescent intensity at 430 nm for the interaction of tamoxifen analogues and W-7 with calmodulin

Compound	% Fluorescent intensity relative to control at 1 μM	3 μM	5 μM
W-7	80	47	26
	0.2 μM	0.6 μM	1 μM
1 (*trans* tamoxifen)	128	157	200
cis tamoxifen	126	160	205
idoxifene	137	183	300
2	113	127	136
3	107	115	120
4	120	145	170
5	130	165	210

Each result is the mean of triplicate determinations which were within 10%.

concentration of 0.5% dimethyl sulphoxide. Basal samples were prepared as above, but contained 1 mM EGTA in place of the $CaCl_2$. The emission intensity was measured at 430 nm with excitation at 360 nm. The fluorescence of the basal samples was subtracted from the test samples in order to obtain the relative fluorescent intensity.

Estrogen receptor binding assay. The affinities of compounds **2, 3, 4** and idoxifene for the estrogen receptor were measured using a competitive binding assay [19]. The cytosol from uteri of immature rats was incubated at 4° for 16 hr with 5 nM 17β-[2,4,6,7-^{3}H]estradiol in the presence of increasing amounts (0.1–100,000 nM) of test compound or unlabelled estradiol. The non-specific binding was quantified by a parallel set of tubes containing 200-fold excess (with respect to [^{3}H]estradiol) of diethylstilboestrol. Unbound compounds were removed with dextran coated charcoal and the receptor-bound [^{3}H]-estradiol was determined. The concentrations of estradiol and test compound required to achieve 50% inhibition of [^{3}H]estradiol binding gives the relative binding affinity (RBA), which is IC_{50} estradiol divided by IC_{50} test compound multiplied by 100. The RBA values for the other compounds in Table 1 were as previously determined [20].

RESULTS

Table 1 displays the IC_{50} values for the inhibition of the calmodulin dependent cAMP phosphodiesterase activity amongst the varied tamoxifen analogues studied. None of these compounds gave any significant inhibition of the calmodulin-independent, or basal, phosphodiesterase activity when assayed at final concentrations of 10 and 20 μM in the presence of 1 mM EGTA. Therefore the tamoxifen derivatives only inhibit the Ca^{2+}/calmodulin activation and have no direct effects on the enzyme itself. A detailed kinetic analysis of the inhibition exerted by compound **4** is shown in the Lineweaver–Burk plot (Fig. 3). The data points from the Lineweaver–Burk plot were analysed using the Enzpack 3 program and the inhibition was found to be competitive with respect to calmodulin. Similar results were obtained with tamoxifen and compound **5** (graphs not shown). The dissociation constants (K_i values) generated from these results are in good agreement with the IC_{50} values and with the published value for tamoxifen, which was shown to be a competitive inhibitor of the calmodulin activation of phosphodiesterase, with a K_i of 0.96 μM [1].

The experiments with the fluorescent probe, TNS, were carried out to confirm that the tamoxifen derivatives are directly interacting with calmodulin. Although TNS (20 μM) on its own has negligible fluorescence, in the presence of 2 μM calmodulin, a 5-fold increase in fluorescence intensity at 430 nm resulted (results not shown). This increase could be abolished by 1 mM EGTA, demonstrating that Ca^{2+} is required in this process. In addition, the calmodulin antagonist, W-7, causes a concentration-dependent decrease in fluorescence produced by the formation of the TNS-Ca^{2+}/calmodulin complex (Table 2). These results are supported by the previous reports [12, 13]. With the tamoxifen derivatives, the basal samples (containing EGTA) displayed negligible fluorescence, although at high concentrations of the antiestrogens there was a small increase in the fluorescence of TNS, possibly due to the lipophilic nature of the compounds. All of the compounds produced a concentration-dependent increase in fluoresence of the TNS-Ca^{2+}/calmodulin complex (Table 2). The most potent inhibitor of the calmodulin dependent phosphodiesterase enzyme, namely idoxifene, with a K_i of 0.33 μM [8], gave the largest increase in fluorescence (3-fold at 1 μM). Tamoxifen, **4** and **5**, with K_i values of 0.83, 1.14 and 0.98 μM against phosphodiesterase, all produced increases in fluorescence of a similar intensity. Surprisingly, although the ring-fused analogue, **3**, did not significantly inhibit the phosphodiesterase enzyme at 50 μM, it did generate a small increase in fluorescence, which may be due to its lipophilicity

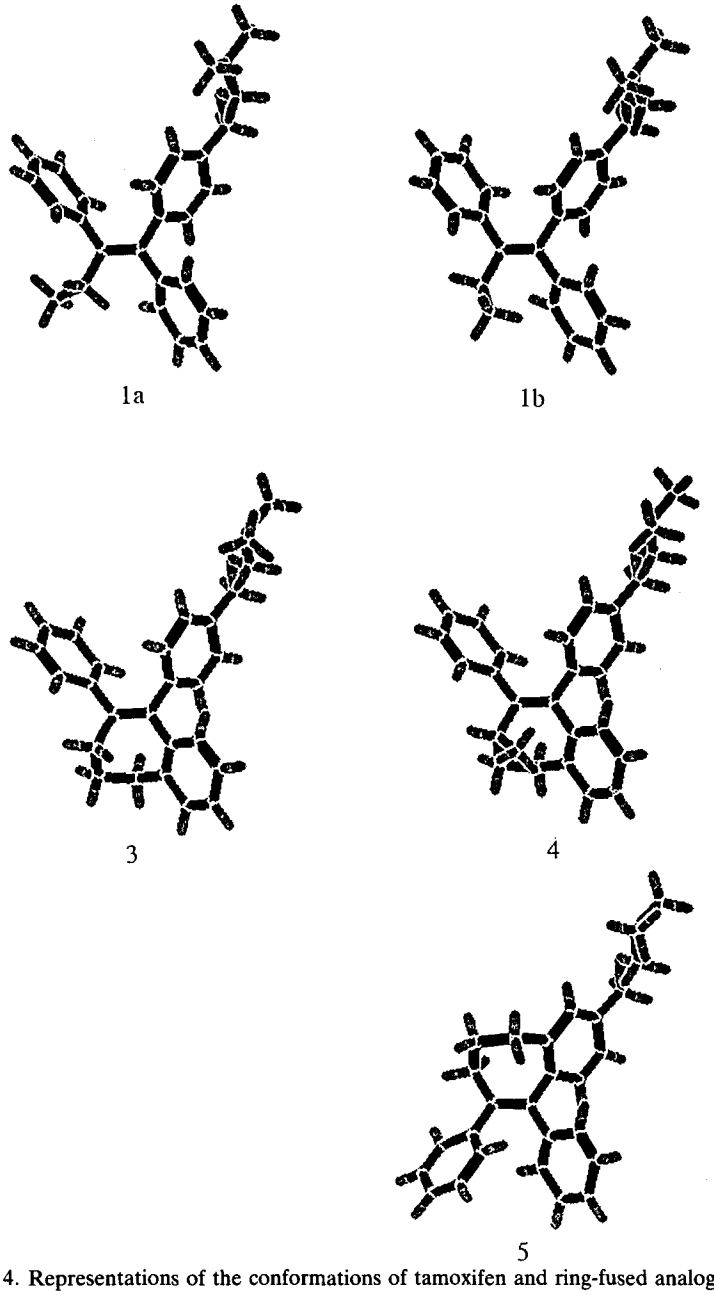

Fig. 4. Representations of the conformations of tamoxifen and ring-fused analogues.

causing non-specific binding to calmodulin. Overall, the results obtained with TNS confirm that these tamoxifen analogues are interacting with calmodulin directly, and the data for the increases in fluorescent intensity agree with the potencies against calmodulin-dependent phosphodiesterase.

This structure–activity study has demonstrated that upon ring fusion of tamoxifen (**1**→**3**), calmodulin inhibition is eradicated, no inhibition of calmodulin-dependent phosphodiesterase being observed at 50 μM. In contrast, however, its isomer **5** corresponding to the "*cis*"-geometric isomer of tamoxifen maintained a similar inhibitory activity as *cis*- and *trans*-tamoxifen. The further compounds synthesised in order to identify the reason for the lack of calmodulin inhibition of **3** were **2** and **4**. Compound **2**, which differs from tamoxifen in having the ethyl-substituent replaced by methyl, had a three-fold reduced calmodulin inhibitory potency. The most revealing result, however, was that of compound **4**, where judicious addition of a methyl group **3**→**4** recovered in the ring-fused analogue nearly all the extent of calmodulin inhibition as found with tamoxifen.

DISCUSSION

The results imply that a requirement for calmodulin

inhibition in the triarylethylene antiestrogens is the presence of a methyl group or similar hydrophobic substituent at a particular region of space that can be occupied by the terminal CH_3 group of the tamoxifen ethyl group, but which remains vacant for the non-inhibitory seven-membered ring analogue **3**.

This structure–activity phenomenon can be explained by considering the conformations that the compounds can adopt. These are depicted in Fig. 4. Two conformations of tamoxifen, **1a** and **1b**, can be considered, both of which have a similar helical arrangement of the out-of-plane aromatic rings but which differ by the orientation of the ethyl group. Conformation **1a** as found in the crystal structure of tamoxifen [21] but **1b** is calculated to have similar energy [22]. In our previous study of ring-fused analogues of tamoxifen [10], a close structural mimicry with tamoxifen was demonstrated when a seven-membered-ring fusion was used; i.e. to give compound **3**. This compound, by X-ray crystallographic analysis of a methoxy precursor, shows a high degree of superimposition with the conformation **1a** of tamoxifen. Given that the ring-fused analogue has affinity to ER like tamoxifen, and the ability to inhibit the growth of estrogen-responsive breast tumour cells *in vitro*, [10, 11] it is likely that conformation **1a** of tamoxifen (or its mirror image) corresponding to the crystal structure is that which binds to the ER. For calmodulin inhibition, tamoxifen is likely to adopt a conformation that is not accessible to the ring-fused analogue **3**, i.e. in a conformation such as **1b** where the methyl group projects frontwards in the orientations shown in Fig. 4. In the ring-fused analogue **3**, the atom corresponding to the methyl group projects rearwards as in Fig. 4, as is the case in tamoxifen conformation **1a**. The deduction from this is that tamoxifen is likely to bind to the estrogen receptor in a different conformation than that which inhibits calmodulin.

In the case of the tamoxifen analogue **2**, the replacement of ethyl by methyl means that the region of space which part of the molecule must occupy for calmodulin inhibition is necessarily vacant. For the structures as drawn in Fig. 3, this region is in front of the plane of the olefinic linkage. For the methylated seven-membered ring compound **4**, the extra methyl group can clearly occupy this region of space and explains why this inhibits calmodulin but **3** does not. The calmodulin inhibitory activity of the *cis*-ring-fused tamoxifen analogue **5** can be explained on the same basis (see Fig. 4). Here, a phenyl ring replaces the space occupied by the ethyl group. Of the two ortho-hydrogens on this ring, one projects frontwards, occupying the region necessary for calmodulin inhibition.

Recently, results of a molecular modelling study used to predict the binding of tamoxifen to calmodulin have been published [22]. Here it is calculated that the preferred orientation of the ethyl group of tamoxifen in its best calmodulin-binding mode is not that observed in the crystal structure of the free drug. This conclusion directly concords with our deductions from experimental data, so we can support the value of the molecular modelling as an approach to give further information on the binding of calmodulin inhibitors. Regarding the ring-fused analogue **3**, this has been shown to behave as an effective suppressor of estrogen-induced tumour growth, despite the absence of calmodulin antagonism. Therefore calmodulin inhibition is not an essential requirement for antitumour action against such cells. That is not to say that calmodulin inhibition does not give benefit: it might be important in determining the cytotoxic (as opposed to cytostatic) action at higher doses which could be significant in providing an irreversible destruction of cancer cells not observed in tests used so far.

The ring-fused compounds employed are displaying still further utility as tools in unravelling the mechanisms of inhibition of tumour cell growth by antiestrogens. We now have a constrained analogue, **3**, that is a "clean" antiestrogen inasmuch as it lacks the calmodulin side activity, together with a closely related derivative, **4**, that by virtue of its conformational constraint should prove valuable for further probing the mode of binding to calmodulin. It is noteworthy that compound **4** still shows binding to the estrogen receptor which can be explained by its conformation having features of both the conformations **1a** and **1b** of tamoxifen. Of particular interest is that compound **4** is chiral, existing as non-superimposable mirror image forms which are likely to differ in the direction of wind of the helix formed by the three phenyl rings induced by the direction of the exocyclic methyl group. These will certainly have different activity, which should yield useful three-dimensional structure–activity data as well as assist rational design of potent calmodulin inhibitors and estrogen receptor antagonists. The separation of the enantiomers of **4** should prove a worthwhile pursuit.

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