



HAL
open science

A potent and selective photoaffinity probe for the anti-estrogen binding site of rat liver.

Marc E. Poirot, Catherine Chailleux, Annick Fargin, Francis Bayard,
Jean-Charles Faye

► To cite this version:

Marc E. Poirot, Catherine Chailleux, Annick Fargin, Francis Bayard, Jean-Charles Faye. A potent and selective photoaffinity probe for the anti-estrogen binding site of rat liver.. *Journal of Biological Chemistry*, 1990, 265 (28), pp.17039-17043. inserm-00090798

HAL Id: inserm-00090798

<https://inserm.hal.science/inserm-00090798v1>

Submitted on 6 Dec 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

A Potent and Selective Photoaffinity Probe for the Anti-estrogen Binding Site of Rat Liver*

(Received for publication, May 14, 1990)

Marc Poirot‡§, Catherine Chailleux‡, Annick Fargin¶, Francis Bayard‡, and Jean-Charles Faye‡||

From the ‡Laboratoire d'Endocrinologie Expérimentale, CHU Rangueil, Bât. L3, Chemin du Vallon, 31054 Toulouse-Cédex, France and the ¶Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The anti-estrogen binding site (ABS) is an apparently ubiquitous component of cells that has been shown to be intimately linked with the antiproliferative effects of certain antiestrogenic compounds, like tamoxifen, which is currently used for the treatment of breast cancer. However, the identification and *in vitro* study of this novel protein has been hampered to date by a lack of convenient probes that will efficiently label the molecule in nonpurified preparations. Thus, using a selective ABS ligand (4-benzylphenoxy-*N*-ethylmorpholine, MBPE) as starting material, we synthesized a photosensitive azido derivative, [(2-azido-4-benzyl)phenoxy]*N*-ethylmorpholine (azido-MBPE) that can be prepared in a tritiated form. Azido-MBPE has a high affinity for ABS ($K_d = 3$ nM), identical to that of tamoxifen, and covalently labels 5 and 12% of membrane-bound and detergent-solubilized ABS, respectively. Its incorporation is selectively and competitively inhibited by other ABS ligands (tamoxifen > nitromifen > hydroxytamoxifen). [³H]Azido-MBPE potently photolabels either membrane-bound or detergent-solubilized ABS as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions revealing specific photoincorporation in a protein band of $M_r = 40,000$. This molecular weight is approximately two times lower than what we observed previously for ABS preparations studied under nondenaturing conditions and postlabeled with [³H]tamoxifen ($M_r = 80,000-110,000$). In chromatofocusing experiments with photolabeled ABS, a single specifically labeled protein fraction migrating with a pI of 6.4 was found to exhibit a M_r of 40,000 when subsequently electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. These results indicate that [³H]azido-MBPE is a specific high affinity probe of ABS that will prove useful in the ultimate identification of this protein.

tamoxifen competes for the binding of estradiol at its receptor, where it acts as an antagonist (2). This mechanism has been proposed to adequately account for tamoxifen's blockade of the proliferative effects of estrogens on "estrogen-dependent" tumors. However, the antiproliferative effects of tamoxifen on cancer cells lacking estrogen receptors (3), suggest a more complex mode of action. Among the multiple proteins that have been reported to specifically bind [³H]tamoxifen, only the antiestrogen binding site (ABS)¹ does so with an affinity comparable with that of the estrogen receptor (*i.e.* $K_d = 3$ nM). We demonstrated previously (4, 5) that ABS, which is a membrane-bound protein mainly located in the microsomal fraction of virtually every cell studied, can be efficiently solubilized from rat tissues in an active state, enabling the study of its physicochemical properties under nondenaturing conditions. We also reported on the synthesis and characterization of a series of compounds based on the structure of *N,N*-diethylphenylmethylphenoxyethanamine, an ABS-specific ligand derived from tamoxifen (6), which likewise binds to ABS but not to estrogen receptors. However, the lack of sufficiently selective covalent probes has, up to now, hindered more stringent biochemical characterization of ABS as well as its putative role in the antiproliferative action of tamoxifen. Thus, to further characterize ABS, the identity of which still remains unknown, we selected one of the above compounds (MBPE) as a precursor in the design of an irreversibly binding ligand for ABS, azido-MBPE. We describe the properties of this potent and selective probe, the first of its kind, which can be prepared in a tritiated form. The covalent incorporation of [³H]azido-MBPE in crude membrane-bound and detergent-solubilized ABS from rat liver has allowed us to determine the isoelectric point and the molecular weight of ABS. Our results underline the usefulness of azido-MBPE in the further characterization and ultimate identification of this novel protein, and in establishing the role of ABS in tumorigenesis.

EXPERIMENTAL PROCEDURES²

Chemicals

[³H]Tamoxifen (specific activity 86 Ci/mmol) was from Du Pont-New England Nuclear, and unlabeled steroids were from Sigma with

The usefulness of tamoxifen (Nolvadex ND) in breast cancer therapy has become well established in recent years (1). However, the exact mechanism of its antiproliferative action is still a matter of some controversy. It has been reported that

* This work was supported in part by grants from the Fondation pour la Recherche Médicale, the Institut National de la Santé et de la Recherche Médicale, Université Paul Sabatier de Toulouse, and the Ligue Française contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a fellowship from the Ligue Française Contre le Cancer.

|| To whom correspondence should be addressed.

¹ The abbreviations used are: ABS, antiestrogen binding site; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MBPE, 4-benzylphenoxy-*N*-ethylmorpholine; azido-MBPE, [(2-azido-4-benzyl)phenoxy]*N*-ethylmorpholine; Tx, tamoxifen; CI 628, nitromifen; OHTx, hydroxytamoxifen; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; bis-Tris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

² Portions of this paper (including part of "Experimental Procedures," and Figs. 1-6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

the exception of tamoxifen which was a gift from Imperial Chemical Industries. Tris, KCl, NaN_3 , glycerol, EDTA, propanediol, and dimethylformamide were from Merck. Monothioglycerol, β -mercaptoethanol, taurine, Tween 20, and SDS were from Sigma, iminoacetic acid was from Aldrich, and CHAPS and bis-Tris were from Serva. Azido-MBPE and [^3H]azido-MBPE were synthesized as described in the Miniprint Section and controlled for chemical and radiochemical purity.

Buffers

Buffers and their composition were as follows: buffer A, homogenization buffer, 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1.5 mM EDTA, 0.01% NaN_3 ; buffer B, nondenaturing extracting buffer, buffer A without sucrose and containing 20 mM CHAPS and 0.4 M KCl; buffer C, binding studies buffer, buffer B readjusted to 2 mM CHAPS and containing 10% glycerol; buffer D, electrophoresis sample buffer, 50 M Tris, pH 6.8, 8% glycerol, 2% SDS, and 2% β -mercaptoethanol; buffer E, elution gel buffer, 50 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 0.1 M EDTA, 12 mM monothioglycerol, and 0.1% SDS; buffer F, chromatofocusing start buffer, 25 mM bis-Tris, iminodiacetic acid, pH 7.1, 1% taurine, and 1% Tween 20; buffer G, chromatofocusing elution buffer, 10% polybuffer 74, iminoacetic acid, pH 4.1, 1% taurine, and Tween 20.

Preparation of Rat Liver Microsomes and Solubilization

Sprague-Dawley rats were ovariectomized by the dorsal route at least 3 weeks before being killed by cervical dislocation. All experiments were carried out at 4 °C except where indicated. Rat livers were perfused *in situ* with physiological serum, removed, and homogenized in 4 volumes of buffer A. Subcellular fractionation was performed as described by Watts and Sutherland (7). The solubilization procedure has been reported previously (4). Briefly, the pellet corresponding to the microsomal fraction was homogenized by sonication in buffer B and the suspension was immediately centrifuged at $100,000 \times g$ for 1 h to eliminate nonsolubilized material. The corresponding supernatant, or soluble fraction, was immediately diluted 1/10 in buffer C to reduce the CHAPS concentration and to stabilize ABS activity. The protein concentration at this step was routinely: 0.4 mg/ml.

Binding Studies

Binding studies were routinely performed with 100- μl aliquots of soluble material, [^3H]tamoxifen, or [^3H]azido-MBPE were incubated in the presence and absence of various concentrations of unlabeled compounds, in the presence of 1 μM estradiol to block ligand binding to estrogen receptors. Following incubation (16 h at 4 °C), bound and free radioligands were separated on Sephadex LH-20 columns (4). Nonspecific binding was determined in the presence of 5 μM tamoxifen and its value corrected according to the method of Rosenthal (8). The inhibition constant (K_i) of azido-MBPE was determined by competition of [^3H]tamoxifen binding (1 and 3 nM), using Dixon plot analysis (9). The dissociation constant (K_d) of [^3H]azido-MBPE (0–100 nM) was established by saturation binding.

Photolysis and Photolabeling

Soluble Preparations—Soluble preparations were incubated for 18 h at 4 °C with 10 nM [^3H]tamoxifen in the presence of 100 nM azido-MBPE. After removing free ligand by filtration on PD10 columns (Bio-Rad) equilibrated with buffer C, the samples were UV-irradiated at 302 nm (4 \times 15 watts) for various periods of time, under a Pyrex[®] glass plate to filter out wavelengths <300 nm. The residual [^3H]tamoxifen binding capacity was measured as described above, following the irradiation period. As a control, the experiment was performed in the absence of azido-MBPE. For the photolabeling experiments, soluble material was processed as described above except that the incubation was performed with 20 nM [^3H]azido-MBPE in presence and absence of a 100-fold excess of unlabeled tamoxifen.

Membrane Preparations—Membrane preparations were only used for photolabeling experiments. Routinely, 500 μl of the membrane preparation (0.6 mg/ml) was incubated in the presence of 20 nM [^3H]azido-MBPE either alone or together with a 100-fold excess of tamoxifen for 18 h at 4 °C. The samples were then filtered over Whatman GF/A glass fiber filters and the filters washed twice with 5 ml of buffer A without sucrose. Following UV irradiation, the filters were incubated for 2 h in extracting buffers D or F for subsequent electrophoresis and chromatofocusing studies, respectively.

SDS-Polyacrylamide Gel Electrophoresis

Irradiated soluble material or irradiated membranes (retrieved from GF/A filters after extraction in buffer D) were precipitated with 10% trichloroacetic acid for 4 h at 4 °C, and centrifuged at $10,000 \times g$ for 20 min. The corresponding pellet was washed twice with ethanol-ether (1:2, v:v), dried, and solubilized in buffer D (final protein concentration = 2.5 mg/ml). SDS-PAGE was performed under denaturing conditions according to the method of Laemmli (10). Briefly, 400- μg protein samples were loaded on 12% slab gels (1.5 mm thick). Following electrophoresis, gels were cut in 5-mm slices which were crushed and eluted for 24 h at room temperature with 2 ml of buffer E. One ml of the supernatant was used for radioactivity counting.

Chromatofocusing

Irradiated membranes were solubilized in buffer F and loaded (6 ml, ≈ 30 mg of protein) on a 30-ml column of PBE 94 resin (Pharmacia LKB Biotechnology Inc.) equilibrated in buffer F. Elution (flow rate: 36 ml/h) was with polybuffer 74 (Pharmacia), and 4-ml fractions were collected. Three ml of each fraction was used for pH determination and 1 ml for radioactivity counting. The usual detergent solutions (buffers B and C), containing CHAPS and KCl, could not be used because of the intolerance of chromatofocusing procedure with salt and the interference of CHAPS with the UV detector in reason of its absorption properties.

RESULTS

Interaction of Azido-MBPE with Rat Liver ABS—For convenience, detergent-solubilized preparations of ABS were used to characterize the binding properties of azido-MBPE and to determine the optimal conditions for its covalent linkage to ABS. Fig. 2 shows that azido-MBPE competitively inhibits the specific binding of [^3H]tamoxifen to ABS and yields a K_i of 3 nM. The saturable and high affinity binding of azido-MBPE to ABS was further confirmed using [^3H]azido-MBPE (Fig. 3, $K_d = 3.2 \pm 0.8$ nM, $n = 6$).

The ability of azido-MBPE to photochemically inactivate soluble ABS was investigated and the results are shown in Fig. 4. The detergent-solubilized preparation was first incubated with azido-MBPE and then eluted through a PD10 column to eliminate free ligand prior to UV irradiation. The progressive decrease of the residual [^3H]tamoxifen binding activity following this treatment indicates that the photoinactivation process is time-dependent. No light-induced damage of ABS could be detected with control preparations until 30-min exposure. In addition, the protein migration pattern of membrane preparations following SDS-PAGE indicated that a 30-min UV exposure did not lead to photolysis (data not shown).

Photolabeling ABS with [^3H]Azido-MBPE:SDS-PAGE—Fig. 5 illustrates the specific photolabeling of solubilized (Fig. 5A) and membrane-bound proteins (Fig. 5B) by [^3H]azido-MBPE following a 30-min exposure to UV light. Electrophoresis on 12% SDS-polyacrylamide gels revealed the incorporation of radioactivity in a protein band corresponding to a molecular mass of ≈ 40 kDa for both preparations. This incorporation could be blocked by tamoxifen as well as by other ABS ligands (tamoxifen > nitromifen > hydroxytamoxifen, data not shown). In the case of membrane preparations, a second minor peak corresponding to a molecular weight of about 53,000 was detectable, but the radioincorporation of [^3H]azido-MBPE was not blocked by tamoxifen (Fig. 5B). Inclusion of protease inhibitors (leupeptin 10 mM, phenylmethylsulfonyl fluoride 0.5 mM, aprotinin 2 mg/ml) in the reaction mixture did not alter this pattern (data not shown). By comparison of the amount of radioactivity incorporated in the $M_r = 40,000$ protein band to the actual ABS concentration in the preparation measured by [^3H]tamoxifen binding, we determined that the efficiency of photolabeling was around 12% for solubilized and 5% for membrane-bound ABS.

Chromatofocusing—The isoelectric point of photolabeled membrane-bound ABS was determined by chromatofocusing, after solubilization in a solution containing Tween and taurocholate (buffer F). The only protein fraction which was protected by tamoxifen from photoincorporation of [³H]azido-MBPE migrated at pH 6.4 (Fig. 6). This fraction, when submitted to SDS-PAGE, exhibited the expected molecular mass of 40 kDa.

DISCUSSION

Photoaffinity probes have proved indispensable for the study of reversible biological systems, since they conserve ligand-receptor interaction by covalent linkage. This technique has for example, resulted in a rapid progression of studies concerned with the purification, the identification, and the subcellular localization of steroid receptors (11), photoprobes have also allowed the purification, the cloning, and the determination of binding site of α_2 -adrenoceptor (12) and they were used to demonstrate the interaction of β_1 -adrenoceptor with G-protein (13).

In the present work evidence has been presented which shows azido-MBPE to be a potent and specific photoaffinity probe for the ABS protein. The identification of ABS is a crucial issue; 1) this is the only protein, besides the estrogen receptor, that displays a high affinity for tamoxifen; 2) tamoxifen is currently used for breast cancer therapy, and we, therefore, must be able to evaluate the consequences of tamoxifen binding to this target protein which is widely expressed in many tissues; and 3) a number of observations subserve the hypothesis that ABS participates to the antiproliferative effect of tamoxifen (1, 3–7). Recently, we have reported an efficient solubilization procedure of ABS from rat uterus microsomes (4), which has been adapted to rat liver in the present study, mostly because this tissue contains the highest ABS concentration (14, 15). We present the synthesis and the characteristics of a photoactivable ligand (azido-MBPE) whose structure was derived from an ABS selective ligand (6). The tritiated compound was used to determine some physicochemical characteristics of ABS. [³H]Tamoxifen competition binding studies, as well as saturation binding at equilibrium with [³H]azido-MBPE, demonstrate that azido-MBPE displays a high affinity for ABS ($K_i = K_d = 3$ nM). Thus, the low specific activity of the tritiated compound (2.2 Ci/mmol) is counterbalanced by its high affinity. Under

exposure to UV light, [³H]azido-MBPE is shown to photoincorporate into both membrane-bound and solubilized ABS with efficiencies of photolabeling (5–12%) over the range of expected for an azido-compound used in crude preparations (16). This photoreactive probe has allowed the determination of the ABS isoelectric point $pI = 6.4$. When analyzed by SDS-PAGE, photolabeled ABS displays a molecular mass of 40 kDa. Since this molecular mass is smaller than what we determined previously in nondenaturing conditions (80–110 kDa, Ref. 4), it is tempting to propose that the 40-kDa peptide represents the simple binding site subunit of a more complex native structure. Combining SDS-PAGE and chromatofocusing analysis, we demonstrated that [³H]azido-MBPE is a potent tool that could be used for subsequent molecular characterization as well as purification of ABS.

Acknowledgments—We wish to thank Dr. M. Hnatowich for his critical reading of the manuscript and his valuable advice. We also thank G. Tatinian and R. Baritaud for technical assistance.

REFERENCES

- Breast Cancer Trials Committee (Edinburg) (1987) *Lancet* **ii**, 171–175
- Jordan, V. C. (1984) *Pharmacol. Rev.* **36**, 245–276
- Chouvet, C., and Saez, S. (1984) *J. Steroid Biochem.* **21**, 755–761
- Fargin, A., Faye, J.-C., le Maire, M., Bayard, F., Potier, M., and Beauregard, G. (1988) *Biochem. J.* **256**, 229–236
- Faye, J.-C., Fargin, A., Valette, A., and Bayard, F. (1987) *Horm. Res. (Basel)* **28**, 202–211
- Fargin, A., Bayard, F., Faye, J.-C., Traore, M., Poirot, M., Klæbe, A., and Perie, J. J. (1988) *Chem. Biol. Interact.* **66**, 101–109
- Watts, C. K. W., and Sutherland, R. L. (1984) *Biochem. Biophys. Res. Commun.* **120**, 109–115
- Rosenthal, R. E. (1967) *Anal. Biochem.* **20**, 522–532
- Dixon, M. and Webb, E. (1964) in *Enzymes* 2nd ed. Longmans Green and Co, London 327–331
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Sweet, F., and Murdock, G. L. (1987) *Endocr. Rev.* **8**, 154–184
- Kobiliska, B. K., Matsui, H., Kobiliska, T. S., Young-Feng, T. L., Francke, U., Caron, M. G., Lavin, T. N., Lefkowitz, R. J., and Regan, J. W. (1987) *Science* **238**, 650–656
- Shorr, R. G. L., Strohsacker, M. W., Lavin, T. N., Lefkowitz, R. J., and Caron, M. G. (1982) *J. Biol. Chem.* **257**, 12341–12350
- Sutherland, R. L., Murphy, L. C., Roo, M. S., Green, M. D., and Whybourne, A. M. (1980) *Nature* **288**, 273–275
- Sudo, K., Monsma, F. J., Jr., and Katzenellenbogen, B. S. (1983) *Endocrinology* **112**, 425–434
- Niznik, H. B., Jarvie, K. R., Bzowej, N. H., Seeman, P., Garlick, R. K., Miller, J. J., Jr., Baidur, N., and Neumeyer, J. L. (1988) *Biochemistry* **27**, 7594–7599

SUPPLEMENTAL MATERIAL
TO
A POTENT AND SELECTIVE PHOTOAFFINITY PROBE FOR THE ANTIESTROGEN
BINDING SITE OF RAT LIVER

MARC POTROT, CATHERINE CHATELLEUX, ANRICK FARGIN, FRANCIS BAYARD,
AND JEAN-CHARLES FAYE

CHEMICAL MATERIALS AND METHODS

Reagents and solvents were of analytical reagent grade and were purchased from Aldrich, Merck and Rathburn. They were used as purchased. Analytical thin layer chromatography was carried out on Merck DC Alufolium Kieselgel 60 F₂₅₄; reverse phase. High pressure liquid chromatography was performed on a Ultrasphere C₁₈ column with a "Système Gold" (Beckman) which comprises an IBM-AT, a 126 solvent module and a 167 scanning detector module operating at 230 nm. Melting points were determined on a Fisher-Johns melting point apparatus and are not corrected. Infrared spectra were recorded on a Perkin-Elmer 599 spectrometer. Proton nuclear magnetic resonance spectra (¹H NMR) were obtained at 200 MHz on a Bruker AC 200 spectrometer. Chemical shifts are reported in the δ system of units relative to tetramethylsilane as an internal standard. Mass spectra were obtained on a Variant MAT 311 A mass spectrometer. Microanalytical data were provided by the Microanalytical Service Laboratory of the Ecole Nationale Supérieure de Chimie de the University Paul Sabatier in Toulouse.

Synthesis of azido-MBPE. The synthesis schemes used are shown in fig. 1.

Synthesis of 4-hydroxy-3-nitro-diphenylmethane (nitro-DPM, fig. 1). 4-hydroxydiphenylmethane (5.0 g, 27.2 mmol) was added with stirring to a cold solution of potassium nitrate (3.0 g, 29.7 mmol) in 37% sulfuric acid. The reaction mixture was maintained at 4°C until the suspension became yellow. The solid was washed with water, then dissolved in 50 ml of methylene chloride and washed again with 100 ml sodium chloride (2M) and potassium bicarbonate (2M) solutions. The organic layer was dried over anhydrous magnesium sulfate and the solvent was evaporated under reduced pressure to give 5.17 g (83%) of crude product. Recrystallisation of the product in hot ethanol gave 4.9 g (78%) of yellow needles, m.p. 66-68°C. TLC (silica Hexane/CHCl₃/MeOH 85:10:5) yielded one spot R_f=0.38. Mass spectrum (70 eV) *m/e* (relative intensity) 229. Infrared (KBr) 3244 cm⁻¹ (OH), 1540 and 1330 cm⁻¹ (NO₂). ¹H NMR ((C₂H₅)₂SO) δ in ppm: 4.03(s) benzylic protons (2H), 7.16-7.86 (m) phenyl ring proton (7H), 11.5 (s) hydroxyl proton (1H) exchangeable with ²H₂O.

Anal. calc. for C₁₃H₁₁NO₃
calculated: C, 68.12; H, 4.80; N, 6.11
found: C, 68.09; H, 4.81; N, 6.21.

Synthesis of [(2-nitro-4-benzyl)phenoxy]N-ethylmorpholine (nitro-MBPE, fig. 1). N-Chloroethylmorpholine, HCl (1.6 g, 8.9 mmol) was added to a solution of nitro-DPM (2g, 8.7mmol) and K₂CO₃ (2.1g, 15.4 mmol) in dried dimethylformamide. After stirring overnight the mixture was filtered, poured in water and extracted with ether. The organic layer was washed twice with 0.1 N NaOH, water, and mixed with hydrochloric acid saturated ether. The precipitate was filtered, dried under P₂O₅ and recrystallised with isopropanol/acetone (8:2) to give 2.9g (77%) of colorless needles, m.p. 111-112°C. TLC (silica Benzene/MeOH/CHCl₃; 7.5:2.0:5) yielded one spot R_f=0.58. Mass spectrum (70eV) *m/e* (relative intensity) 342. ¹H NMR ((C₂H₅)₂SO) δ in ppm: 3.47 (m,4) -N(CH₂-CH₂)₂O-, 3.65-3.69 (t, 2, J=4.5 Hz) Ar-O-CH₂-CH₂-N<, 4.01 (m,4) -N(CH₂-CH₂)₂O-, 4.1 (s, 2) O-CH₂-Ar-O-, 4.72-4.76 (t,2,J=4.5Hz) Ar-O-CH₂-CH₂-N<; 7.28-7.49 (m, 6, C₆H₅- + 1 ring proton ortho from -O- and meta from benzylic -CH₂-); 7.66-7.71 (dd,1,ring proton ortho from benzylic -CH₂-, meta from -O- and para from -NO₂, J_o=8.65 Hz and J_m=2.1 Hz); 7.92-7.94 (d,1, ring proton ortho from -NO₂ and from benzylic -CH₂-).

Anal. calc. for C₁₉H₂₂N₂O₄:
calculated: C, 66.67; H, 6.4; N, 8.19
found: C, 66.57; H, 6.34; N, 8.25.

Synthesis of [(2-amino-4-benzyl)phenoxy]N-ethylmorpholine (amino-MBPE, fig. 1). To a refluxing mixture of hydrazine hydrate (1.25 g, 25 mmol), 20 ml of methanol and 150 mg of Raney Ni, a solution of nitro-MBPE (1.5 g, 4.4 mmol) in 20 ml of methanol was added dropwise. The reaction mixture was stirred under reflux for 30 min, then filtered over celite and the solvent evaporated under reduced pressure. Recrystallisation in hot ethanol gave 1.37 g (94%) of colorless needles m.p. 137-138°C. TLC (silica benzene/MeOH/CHCl₃; 7.5:2.0:5) yielded one spot R_f=0.50. Using high performance liquid chromatography a 40-min linear gradient of 0-100% acetonitrile in ammonium carbonate (5mM) pH 8 at a flow rate of 2ml/min gave one UV-absorbing peak at the elution time 26.1 min. Mass spectrum (70eV) *m/e* (relative intensity) 312. Infra-Red (KBr) 3387 and 3304 cm⁻¹ (-NH₂). ¹H NMR δ in ppm: 3.47-4.76 (m,12) ethyl morpholine moiety; 4.81 (s, 2) -NH₂ exchangeable with ²H₂O; 6.45-6.49 (dd, 1, J_o=8 Hz J_m=2 Hz) ring proton ortho from benzylic -CH₂-, meta from -O- and para from -NH₂; 6.57- 6.58 (d, 1, J= 2Hz) ring proton ortho from benzylic -CH₂-, from -NH₂ and meta from -O-; 6.80-6.84 (d, 1, J=8 Hz) ring proton ortho from -O-, meta from -NH₂ and meta from benzylic -CH₂-; 7.24-7.41 (m, 5) C₆H₅-.

Anal. calc. for C₁₉H₂₄N₂O₂

calculated: C, 73.08; H, 7.69; N, 8.97.
found: C, 72.96; H, 7.68; N, 9.01.

Synthesis of [(2-azido-4-benzyl)phenoxy]N-ethylmorpholine (azido-MBPE, fig.1). A solution of amino-MBPE (1g, 3.2 mmol) in 15 ml of acetone and 5 ml of hydrochloric acid (2N) at 0°C was diazotised with a solution of NaNO₂ (0.45 g, 6.5 mmol) in 5ml of water. After 30 min the cold solution of diazonium salt was added to a ten fold excess (2.08 g, 32 mmol) of NaN₃ in 5 ml of water. After additional stirring for 30 min the acidic mixture was basified with a cold solution of 1M K₂CO₃ and then extracted three times with 50 ml of ether. The combined extracts were dried over MgSO₄ and concentrated to dryness to give a yellow oil. The crude product was crystallized in acetone to give 0.45 g (42%) of colorless needles, mp 122-123°C. High performance liquid chromatography analysis (in the same condition than the NH₂-MBPE) gave one UV-absorbing peak eluting at 30.5 min. Infrared (KBr) 2114 cm⁻¹ (strong, azide). Mass spectrum (70eV) *m/e* (relative intensity) 338.

Anal. calc. for C₁₉H₂₂N₄O₂
calculated: C, 67.46; H, 6.51; N, 16.57.
found: C, 67.52; H, 6.55; N, 16.54.

Synthesis of tritiated form of [(2-azido-4-benzyl)phenoxy]N-ethylmorpholine. The tritiation was performed at the Commissariat à l'Energie Atomique (Saclay, FRANCE) by catalytic tritium exchange with ³H₂ gas over PdO/BaSO₄ of the amino-MBPE (50 mg) followed by transformation to the azido group, as described in details above for the unlabeled derivatives. The chemical and radiochemical purity of [³H]azido-MBPE was determined by HPLC as described above for unlabeled compound. A UV-absorbing peak was eluted at 30.5 min, identical to the elution time of the unlabeled azide. The specific activity of this compound was determined to be 2.2 Ci/mmol by quantitative high performance liquid chromatography. The mass of an injected sample was determined by comparing the integrated area of the eluted peak with an area calibration curve generated by injection of known weights of unlabeled azido-MBPE, and the activity in the eluted peak was determined by liquid scintillation counting.

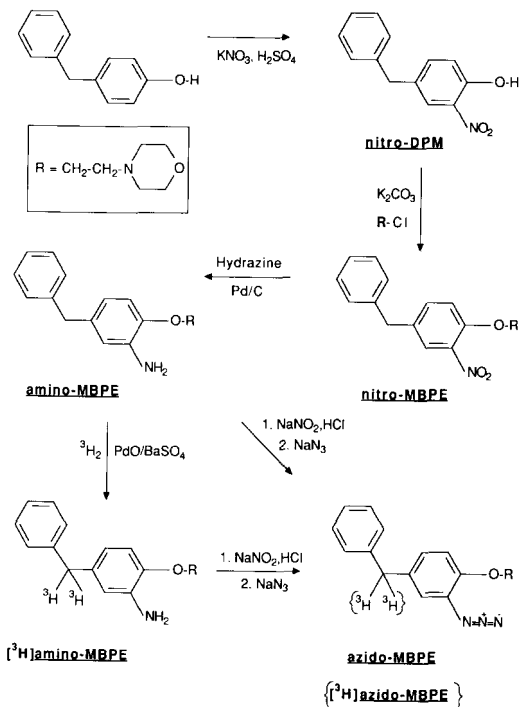


Fig.1: Outline of synthesis of azido-MBPE

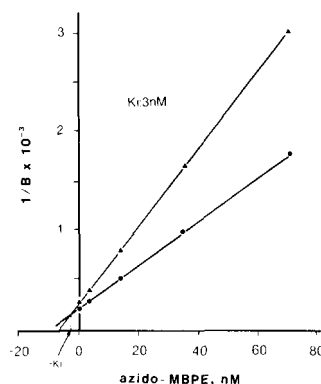


Fig.2: Inhibition of [³H]Tamoxifen binding to solubilized ABS by azido-MBPE. Details are described under "Experimental procedures". The experiment shown is representative of five independent determinations with each data point determined in triplicate. Data are plotted according to Dixon representation (9).

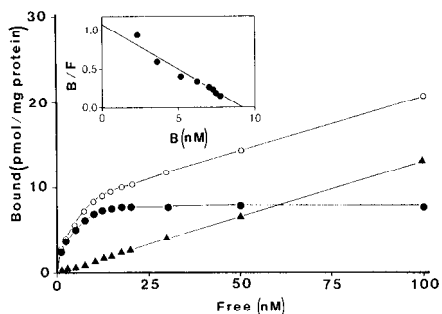


Fig.3: $[^3\text{H}]$ azido-MBPE saturation binding curve to solubilized ABS

Details are as described under "Experimental procedures". 100 μl of detergent extracts were incubated with increasing concentrations of $[^3\text{H}]$ azido-MBPE in the absence (total binding, \circ) and the presence (nonspecific binding, \blacktriangle) of 5 μM tamoxifen. Specific binding (\bullet) is the difference between total and non specific. The graph shown is representative of 6 independent experiments with each data points performed in triplicate. The corresponding Scatchard representation of specific binding is shown in the inset.

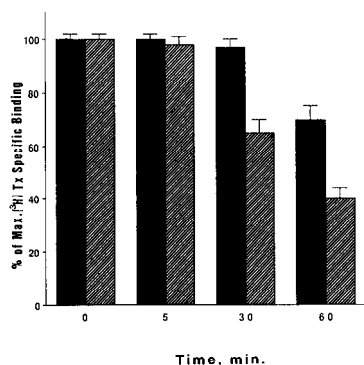


Fig.4: Time course of photoinactivation of solubilized ABS

Solubilized ABS extracts were incubated in the presence (hatched bars) and the absence (black bars) of azido-MBPE (140 nM), for 18 h at 4°C. Free ligands were removed by chromatography on PD10 column before exposure for the indicated periods of time. Following irradiation, the residual ABS concentration was determined by $[^3\text{H}]$ tamoxifen binding. Each point represents the average of three independent determinations \pm standard deviation.

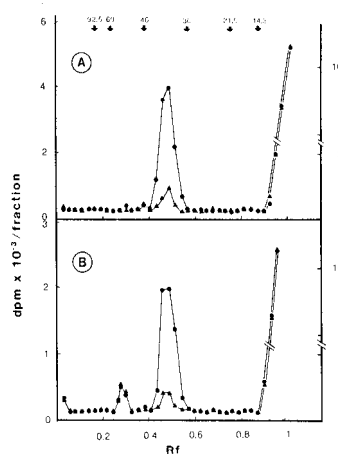


Fig.5: Photolabeling rat liver ABS with $[^3\text{H}]$ azido-MBPE

Detergent extracts (A) or membrane preparations (B) were incubated with 20 nM $[^3\text{H}]$ azido-MBPE alone (\bullet) or together with 5 μM tamoxifen (\blacktriangle). Prior to UV irradiation for 30 min, free ligands were removed by filtration on PD10 columns. Samples were then subjected to SDS-PAGE on 12% gels. Following electrophoresis, the gels were cut in 5 mm slices, the radioactivity counted in each slice, and the values plotted as a function of Rf (distance of migration protein/distance of migration of the dye). The experiment shown is representative of five independent experiments. The only photoincorporation peak which can be blocked by tamoxifen displays a molecular mass of 40 ± 3 kDa ($n = 5$).

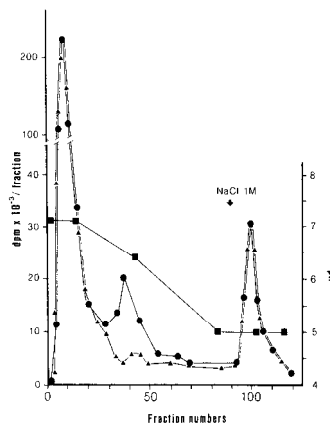


Fig.6: Chromatofocusing of photolabeled membrane-ABS

Membrane preparations were photolabeled as described in the legend of Fig.5. Photolabeled membrane-bound ABS were then solubilized from GF/A filters with buffer F and applied to PBE 94 resin equilibrated in buffer F. Elution was performed with buffer G (see "Experimental Procedures") and radioactivity counted in each fraction (\bullet). The squares represent the pH gradient.