Project Title: MLSCN Assay for Ligands of GPR30 and Classical Estrogen Receptors

Grant Number: 1X01MH077627-01

Screening Center Name: New Mexico Molecular Libraries Screening Center

Principal Investigator of Screening Center: Larry A. Sklar, Ph.D

Assay Submitter and Institution:

Eric R. Prossnitz
University of New Mexico

PubChem Bioassay Identifier (AID): 1210, 1211, 1212, 1213,1219, 1221, 1223, 1224, 1226

Assay Target: GPR30

Probe PubChem Compound Identifier (CID): 3136849

Mode of Action: Competitive inhibitor of ligand binding

Specific Aim: To identify small molecule ligands specific for GPR30 or the classical estrogen receptors (ERα and ERβ).

Significance and Rationale:

Target: Transmembrane Estrogen Receptor GPR30/GPER.

In the late 1990's, an orphan GPCR was cloned [1]. The GPCR identified in these studies displayed low homology to the chemokine family of GPCRs (~30%) and was expressed in numerous tissues throughout the body. In 2000, a potential agonist for this GPCR was identified. GPR30-associated activation of MAP kinases (Erk1/2) by estrogen in breast cancer cell lines was observed [2]. ER antagonists, ICI 182,780 and tamoxifen, at high concentrations, were also capable of mediating Erk activation. Erk activation proceeded through a pertussis toxin-sensitive pathway that involved the transactivation of EGFRs through the release of cell-surface heparin-bound EGF. A follow-up report described a second phase of GPR30-dependent signaling via adenylyl cyclase [3]. More recently, in keratinocytes, GPR30 has been suggested to promote estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting Bcl-2 expression and to promote cell growth by stimulation of cyclin D expression. Furthermore, upregulation of nerve growth factor production in macrophages by inducing c-fos expression has also been demonstrated. The upregulation of c-fos by estrogen and phytoestrogens has also now been shown in breast cancer cells [4].

Estrogen exerts numerous diverse physiological effects, being involved in the growth, development and homeostasis of numerous tissues. The best understood of these are mammalian female reproduction and breast development. In addition, estrogen regulates skeletal physiology, (cardio)vascular function, the central nervous system as well as the immune system. Estrogen stimulation initiates numerous signaling pathways that can lead to various cellular responses from adhesion and migration to survival and proliferation to cardiovascular and neuro-protection to organogenesis to angiogenesis to cancer. In addition, stress responses, feeding patterns, sleep cycles and temperature homeostasis are regulated by estrogen [5].

Known Ligands:

Estrogen (17β-estradiol, E2) is a critical hormone in the human body. Estrogen represents one of a family of steroid hormones, which also includes progesterone, testosterone, cortisol/glucocorticoids and aldosterone/ mineralocorticoids, that control many aspects of mammalian physiology. Additional physiological estrogen agonists, estrone (E1) and estriol (E3), also mediate biological functions.

Estrogen-like activity can be found in a large variety of sources, both natural and manmade. These include phytoestrogens/isoflavinoids, from plants and fungi, as well as xenoestrogens, which include a variety of pesticides, polychlorinated biphenyls and plasticizers. The majority of these compounds are thought to exert their effects through the classical nuclear estrogen receptor(s). However, estrogen receptors unrelated to ERα and ERβ, but associated with membranes, such as GPR30, have been proposed to mediate some of the effects of xenoestrogens. No ligands specific to GPR30 were known at the time of this screening [6].

Assay Description, Implementation and Screening:

PubChem Bioassay Name: GPR30 Binding

List of PubChem Bioassay Identifiers (AIDs): 1212, 1213,1219, 1224

PubChem Primary Assay Description (AIDs): 1212, 1213

In order to minimize the biomolecular screening required, we first employed virtual screening, to rapidly identify structures of interest for biological screening. We began by virtually screening a library of 10,000 in-house molecules (pre-optimized to be enriched in GPCR-binding ligands based on the concept of the GPCR-privileged substructure) using a combination of 2D and 3D similarity approaches. The combined similarity score attributed 40% weight to 2D fingerprints, 40% to the shape-based similarities and 20% to pharmacophore-based similarity. Given this composite score, the top 100 ranked molecules were selected for biomolecular screening.

To accomplish the biomolecular screening, we employed a fluorescently labeled estrogen derivative (an Alexa633 conjugate of 17α-[4-aminomethyl-phenylethynyl]-estra-1, 3, 5(10)-triene-3, 17β-diol) that we have shown binds GPR30 as well as ERα/β. Competitive ligand binding was assessed in transiently transfected COS7 cells, which do not display detectable specific binding of the fluorescent estrogen in the absence of exogenous GPR30 or ER expression. To maximize the bound fluorescent signal, a GPR30-GFP fusion protein was expressed and cells were gated for high levels of green (FL1) fluorescence (i.e. GPR30 expression). As GPR30 expression is localized to the endoplasmic reticulum and not the plasma membrane, cells were permeabilized with saponin to permit access of the charged E2-Alexa to GPR30. Biomolecular screening was carried out using 17β-estradiol as a positive control to block specific binding of E2-Alexa633 to GPR30.

Center Summary of the Primary Screen:

A combination of virtual screening (on ~10,000 compounds) and a biomolecular flow cytometry screening (on the top 100 compounds) was used to identify a novel small molecule inhibitor of estrogen binding to GPR30. Calculation of the Z' factor yielded a value of 0.5-0.7, demonstrating the robustness of the assay. From the primary screen performed at a compound concentration of 10 μM, three compounds resulted in inhibition of fluorescent estrogen binding greater than 60%. These compounds as well as numerous intermediate hits were retested manually from the master compound plate for their ability to inhibit binding of fluorescent estrogen to GPR30, which revealed that only compound 92, designated G-1, consistently inhibited binding. A full description of the discovery and characterization of G-1 has been published in *Nature Chemical Biology* [7].

Probe Optimization:

Secondary Screens:

Cellular activity:

To characterize the binding properties of G-1 in greater depth, we determined the binding affinity to GPR30 and the binding specificity with respect to ERα and ERβ [7]. Competition binding of the fluorescent estrogen by 17β-estradiol in GPR30 expressing cells yielded a Ki of 5.7 nM whereas the Ki for G-1 was 11 nM (**Fig. 1a**). Competition binding in ERα and ERβ expressing cells yielded Ki values for 17β-estradiol of 0.30 and 0.38 nM, respectively with no significant binding of G-1 at concentrations up to 1 μM (**Figs. 1b and c**).

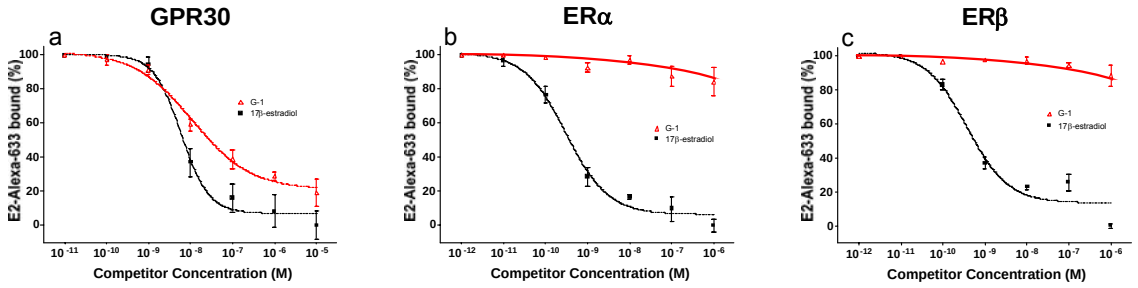


Fig. 1 Specificity of G-1 for GPR30 compared to ERα and ERβ.

We next examined the functional capabilities of G-1 in comparison to estrogen with respect to the rapid mobilization of intracellular calcium. Whereas 17β-estradiol initiates a rapid rise in intracellular calcium concentrations, G-1 produces a slower calcium increase (**Fig. 2a**). The specificity of the response is demonstrated by the fact that G-1 has no effect on control COS7 cells (not expressing GPR30) and that 17α-estradiol has no effect on GPR30-expressing COS7 cells. To confirm the selectivity of G-1 toward GPR30 as compared to ERα and ERβ, we also examined calcium mobilization in COS7 cells expressing either ERα or ERβ. In neither case did G-1 elicit a calcium response (**Figs. 2b and c**). This is in contrast to 17β-estradiol, which yielded a rapid response with both ERα and ERβ. As with

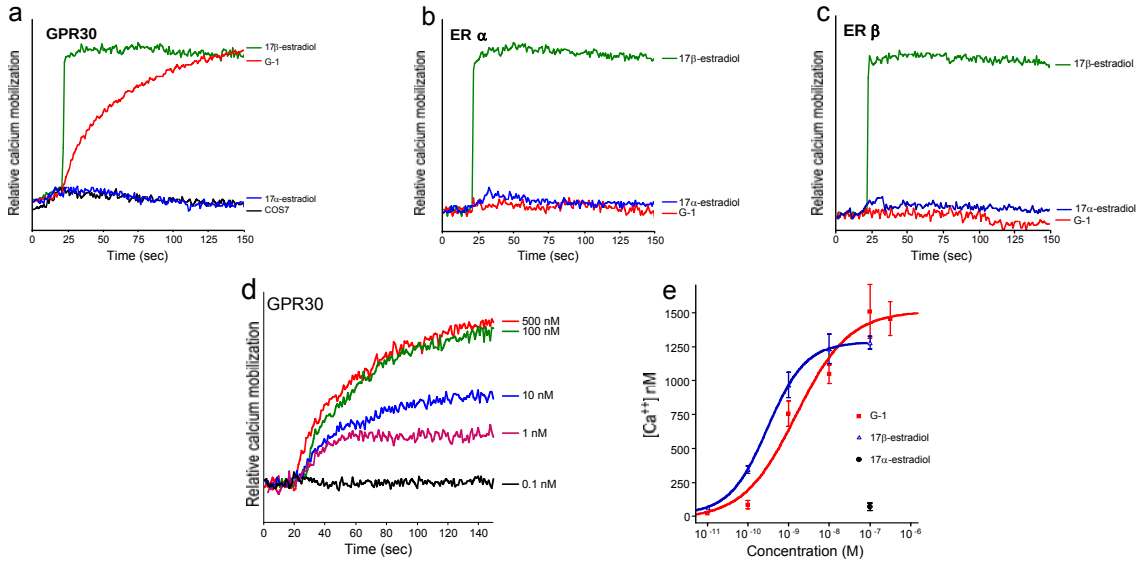


Fig. 2 Agonism of G-1 towards GPR30 but not ERα or ERβ.

GPR30, 17α-estradiol had no effect on ERα or ERβ. Dose-response assays demonstrated that the mobilization of intracellular calcium initiated by G-1 in the presence of GPR30 was concentration-dependent with an EC_{50} of about 4 nM (**Figs. 2d and e**). This compares to an EC_{50} for 17β-estradiol of approximately 0.3 nM.

In addition to calcium mobilization, we have shown that stimulation of ERα, ERβ and GPR30 with estrogen results in PI3K activation, resulting in the nuclear accumulation of PIP3. To validate that G-1 activates a similar complement of signaling pathways as estrogen, we examined the activation of PI3K in receptor transfected COS7 cells. As previously observed, estrogen stimulates the nuclear accumulation of PIP3 through all three receptors. However, as observed for calcium signaling, G-1 selectively activates GPR30 and not ERα or ERβ(**Fig. 3**).
Testing of selected additional compounds containing the 4 ring core structure found in G-1

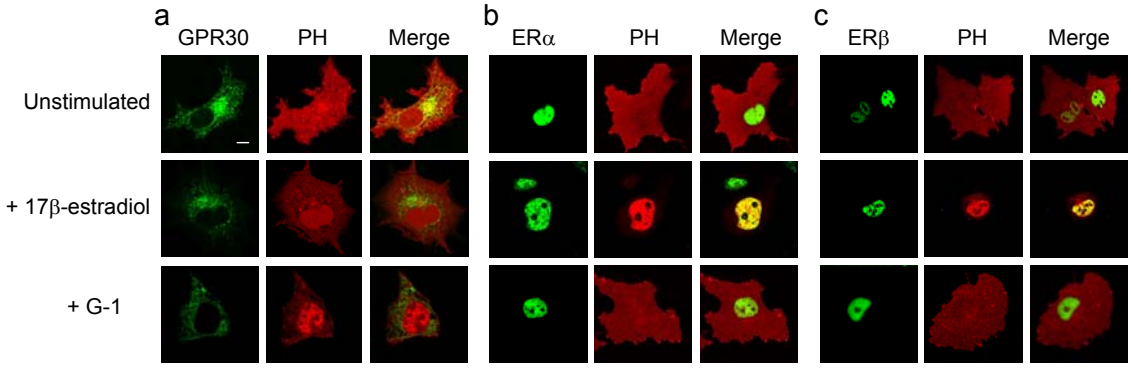


Fig 3. Nuclear PIP3 accumulation resulting from PI3K activation is mediated by G-1 through GPR30 but not ERα or ERβ.

led to the identification of additional compounds with low affinity and minimal specificity for GPR30 and ERs (**Table I**). A number of inactive compounds with respect to all three estrogen receptors were also identified.

Center summary of probe properties and recommendations for the scientific use of probe as research tool:

G-1 has been identified as a highly selective GPR30 agonist (with little to no binding/activity towards classical estrogen receptors ERα/ERβ). It has an affinity for GPR30 of ~10 nM. It is recommended that G-1 be used at concentrations up to 1 μM (lower concentrations are recommended to avoid possible off target effects).

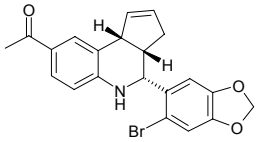
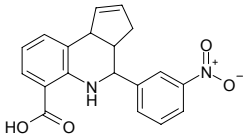
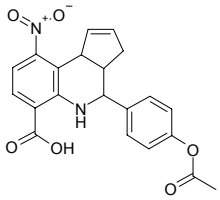
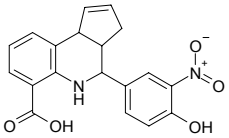
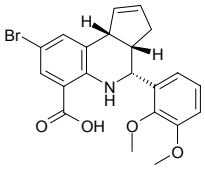
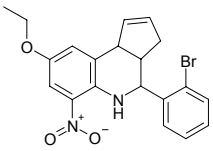
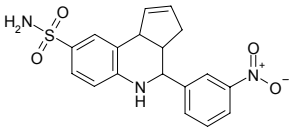
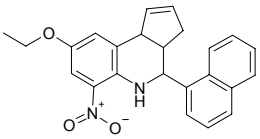
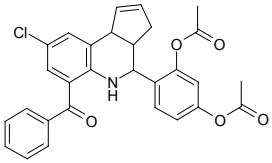
Uses of G-1 as a probe to investigate GPR30-mediated biology:

Albanito, L., Madeo, A., Lappano, R., Vivacqua, A., Rago, V., Carpino, A., Oprea, T.I., Prossnitz, E.R., Musti, A.M., Ando, S., Maggiolini, M. (2007) G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res* **67**, 1859-66.

Brailoiu, E., Dun, S.L., Brailoiu, G.C., Mizuo, K., Sklar, L.A., Oprea, T.I., Prossnitz, E.R., Dun, N.J. (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J. Endocrinol.* **193**, 311-321.

Appendices

Table I.

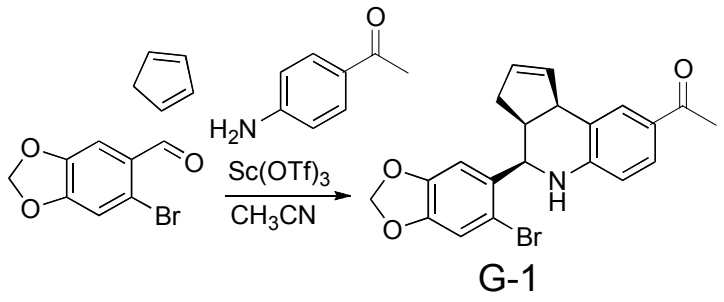
PubChem CID	PubChem SID	Structure	MW	ERα (IC_{50})	ERβ (IC_{50})	GPR30 (IC_{50})
5322399	48409542		412.28	>1.0E-04	>1.0E-04	1.1E-08
2854262	48409568		336.35	8.9E-07	1.5E-05	4.7E-07
2900725	48409587		394.39	1.1E-05	1.4E-05	1.4E-05
4182169	48409588		352.35	6.3E-07	1.6E-05	8.2E-07
5322461	48409599		430.30	6.7E-06	4.2E-05	1.2E-05
2871986	48409583		415.29	ND	ND	ND
2868619	48409569		371.41	ND	ND	ND
3120364	48409579		386.45	ND	ND	ND
3843745	48409584		501.97	ND	ND	ND

ND – no detectable competition at 10 uM competitor

Description of G-1 synthesis

The tetrahydro-3H-cyclopenta[c]quinoline scaffold of G-1 is accessible via the versatile three-component Povarov cyclization, and our original synthesis used trifluoroacetic acid to initiate the reaction of 4-aminoacetophenone, 6-bromopiperonal and cyclopentadiene, followed by preparative HPLC for the isolation of G-1[7]. We evaluated a series of reaction conditions employing other protic and Lewis acid catalysts to optimize reaction rate, yield, and diastereoselectivity in order to provide an improved preparative procedure. Our optimized one-step procedure employing the catalyst $Sc(OTf)_3$ [8] in acetonitrile resulted in rapid reaction times, excellent yield and enhanced selectivity favoring the syn diastereomer as illustrated in **Scheme 1**. Purification by preparative column chromatography gave G-1 in 97% yield as a mixture of syn/anti diastereomers (95/5). The stereochemistry is distinguished by the ^{1}H-NMR coupling pattern of H-4 (4.98 ppm) with a coupling constant of 3.1 Hz that is characteristic for the syn orientation of the cyclopentene ring and phenyl group. The product was fully characterized by NMR spectroscopy in $CDCl_3$, and HPLC-MS with positive ion detection showed the correct molecular ion (MH^+) as well as a single peak by UV (PDA/λ_{max}= 324 nm) detection.

Scheme 1. Synthesis of G-1



Synthesis and characterization of G-1 (1-[4-(6-Bromo-benzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone)

A catalytic amount of $Sc(OTf)_3$ (0.049 g, 0.1mmol, 10 mol%) in anhydrous acetonitrile (1 mL) was added to a mixture of 6-bromopiperanal (0.229 g, 1.00 mmol), 4-aminoacetophenone (0.135 g, 1.0 mmol) and cyclopentadiene (0.33 g, 5.0 mmol) in acetonitrile (3 mL). The reaction was stirred at ambient temperature (~23˚C) for 3 h with monitoring of product formation by thin layer chromatography using 20% ethyl acetate/hexane eluent (R_f 0.3). The volatiles were removed *in vacuo*. The residue was purified by column chromatography using 8% ethyl acetate/hexane to give the product G-1 as a colorless solid (400mg, 97%): mp 103-105° C; FT-IR (**Fig. 1**) (KBr, cm^{-1}) 3324(m), 2895(w), 1659(s), 1596(s), 1474(s). ^{1}H NMR (**Fig. 2**) (300 MHz, $CDCl_3$) δ 7.70 (d, *J* = 1.6 Hz, 1H), 7.61 (dd, *J* = 8.3, 1.6 Hz, 1H) 7.09 (s, 1H), 7.03 (s, 1H), 6.61 (d, *J* = 8.3 Hz, 1H), 6.00 (d, *J* = 1.3 Hz, 1H), 5.99 (d, *J* = 1.3 Hz, 1H), 5.96-5.92 (m, 1H), 5.68-5.66 (m, 1H), 4.98 (d, *J* = 3.1 Hz, 1H), 4.12 (d, *J* = 8.3 Hz, 1H), 4.02 (bs, 1H), 3.23-3.15 (m, 1H), 2.57-2.46 (m, 4H), 1.86-1.77 (m, 1H). ^{13}C NMR (**Fig. 3**) (75 MHz, $CDCl_3$) δ 196.5, 149.9, 147.6, 147.5, 133.8, 133.6, 130.4, 130.0, 128.7, 127.6, 125.1, 115.2, 113.1, 112.9, 107.7, 101.8, 56.2, 45.4, 42.0, 31.4, 26.1.

HPLC-MS: Electrospray positive ion (cone voltage 62 V, Capillary Voltage 3 kV).
G-1 (1mg/mL CH_3CN, 20 μL) was injected onto a Symmetry® C18 (5 μm, 3.0 X 150 mm, Waters) column and eluted with 60-90% CH_3CN in H_2O (gradient 1.5 % min^{-1}) and exhibited

a single peak R_t= 12.42 min. **(Fig 4)**. UV-Vis λ_{max} 324 nm **(Fig. 5)**. ESI-MS *m/z* (ES+) calcd for $C_{21}H_{18}BrNO_3$ $(M+H)^+$ 412.05; found 412.18 **(Fig. 5)**.

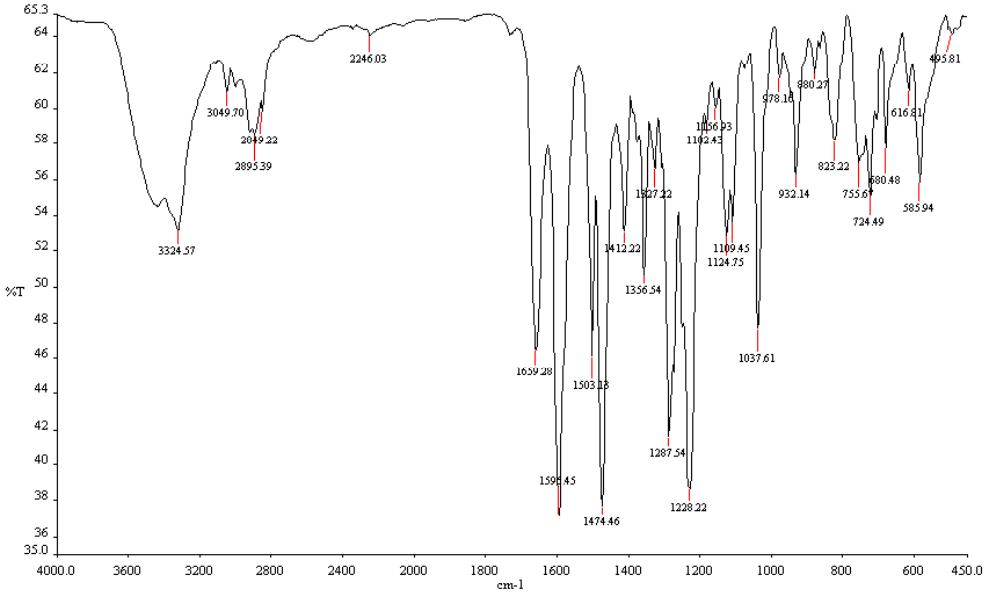


Fig. 1. FTIR Spectrum of G-1

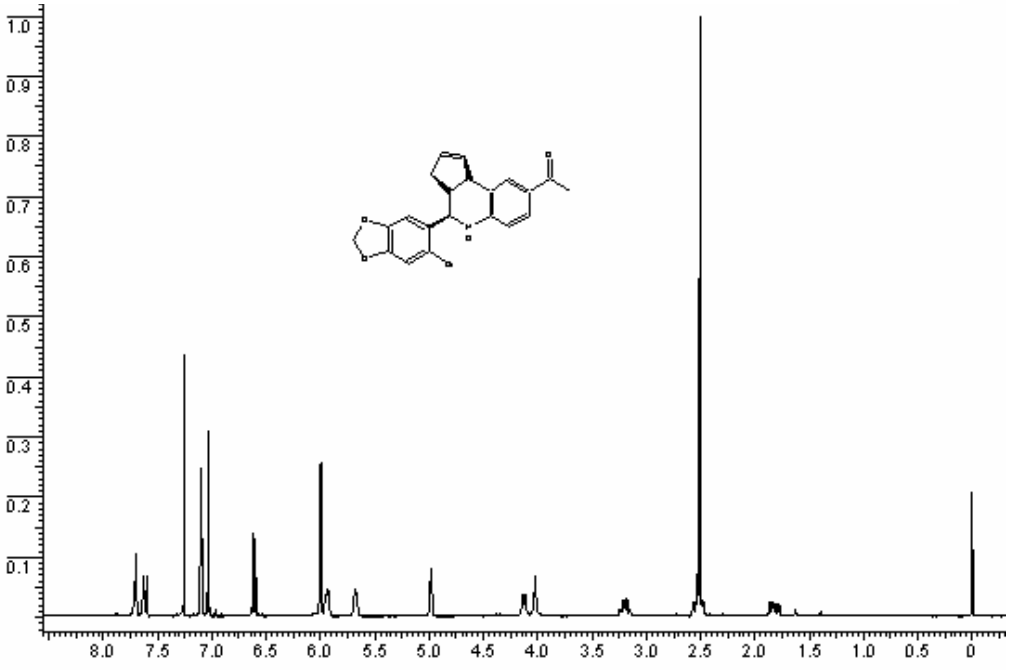


Fig. 2. ^{1}H NMR of G-1

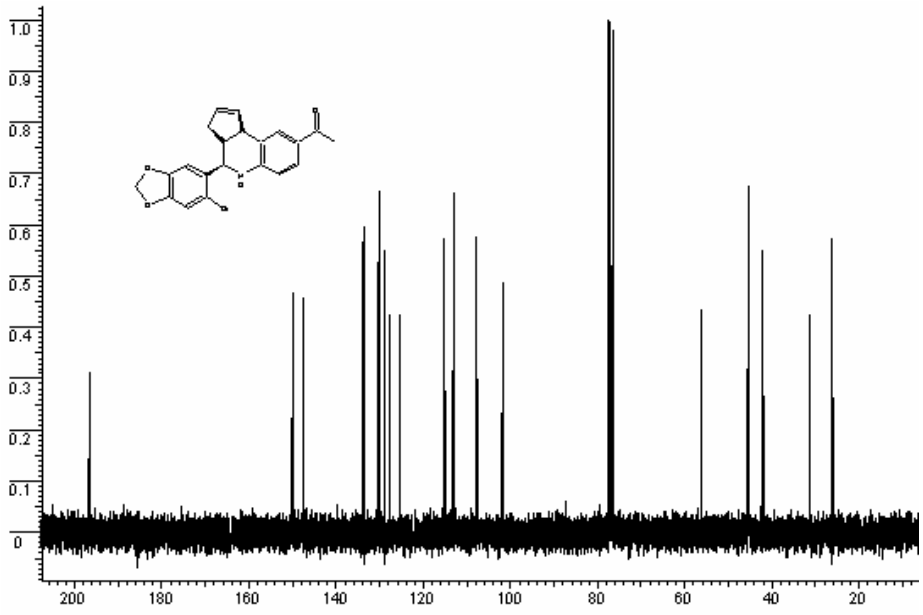


Fig. 3. ^{13}C NMR of G-1

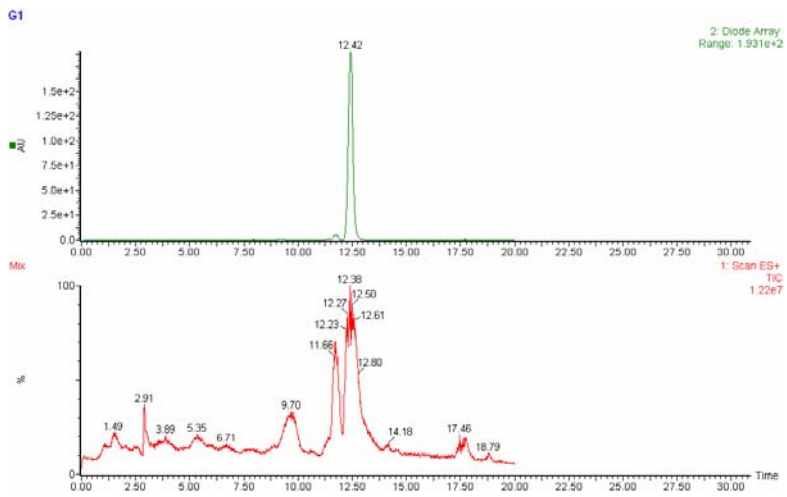


Fig. 4. HPLC PDA (top) and ESI-MS (ES+) TIC (bottom) of G-1

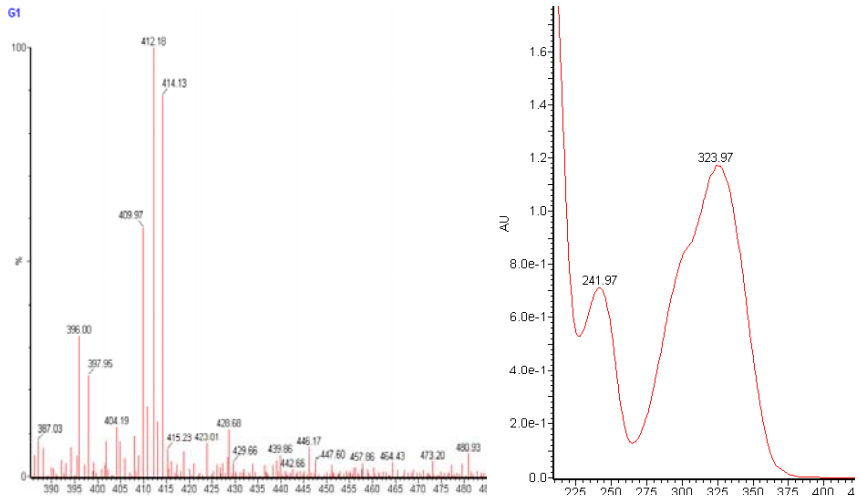


Fig. 5. ESI-MS (left) and UV-Vis (right) of G-1

Bibliography:

1. Owman, C., Blay, P., Nilsson, C., Lolait, S.J. (1996) Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochem Biophys Res Commun* **228**, 285-92.

2. Filardo, E.J., Quinn, J.A., Bland, K.I., Frackelton, A.R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Molecular endocrinology (Baltimore, Md* **14**, 1649-60.

3. Filardo, E.J., Quinn, J.A., Frackelton, A.R., Jr., Bland, K.I. (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Molecular endocrinology (Baltimore, Md* **16**, 70-84.

4. Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A.G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A.M., Picard, D., Ando, S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* **279**, 27008-27016.

5. Edwards, D.P. (2005) Regulation of signal transduction pathways by estrogen and progesterone. *Annu Rev Physiol* **67**, 335-76.

6. Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**, 1625-30.

7. Bologa, C.G., Revankar, C.M., Young, S.M., Edwards, B.S., Arterburn, J.B., Kiselyov, A.S., Parker, M.A., Tkachenko, S.E., Savchuck, N.P., Sklar, L.A., Oprea, T.I., Prossnitz, E.R. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2**, 207-12.

8. Kobayashi, S., Ishitani, H., Nagayama, S. (1995) Lanthanide triflate catalyzed imino Diels-Alder reactions; convenient synthesis of pyridine and quinoline derivatives. *Synthesis*, 1195-1202.