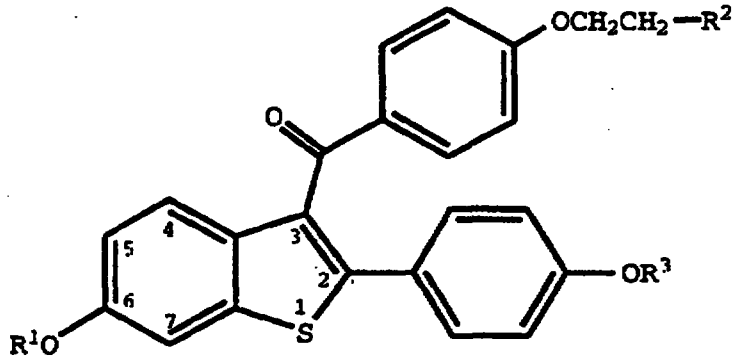
METHODS OF INHIBITING ENVIRONMENTAL ESTROGENS

Estrogen is an endogenous hormone which has a variety of significant functions in human physiology. Predominantly, this hormone affects the reproductive system of both males and females. For example, in the adult female, estrogen is integral in pregnancy and lactation processes. It also lowers the risk of heart attack and osteoporosis. In addition, the ratio of estrogen to androgens in pre-natal and early post-natal stages regulates sexual differentiation and proper formation of the appropriate reproductive organs. Estrogen has also been associated with increases in breast and uterine cell growth, *i.e.* cancer. In males, estrogen regulates sperm production, with abnormally high concentrations resulting in decreases in sperm count.

In recent years, there has been increasing concern over the exposure of human and wildlife populations to manufactured or naturally occuring chemicals which act to mimic the action of endogenous estrogen. These substances are derived from a variety of sources including pesticides, animal health products, plastics, combustion-by-products, and plants (phytoestrogens). Specific examples include zeranol, genistein, coumestrol, o,p-DDT, kepone, methoxychlor, zearalenone, dioxin, p-octylphenol, and bisphenol A. In general, this class of compounds has been termed environmental estrogens and has been implicated in a variety of disease states including gynecomastia (DiRaimondo *et al.*, *N. Engl. J. Med. 302*, 1089, (1980)) precocious puberty (Comas, *Lancet*, 1299, (1982)), low sperm counts (R.M. Sharpe *et al. Lancet, 341*, 1392, (1993)), endometriosis (S. E. Rier *et al., Fundam. Appl. Toxicol., 31*, 423, (1993)), and breast cancer (M.S. Wolff *et al., J. Natl. Cancer Inst., 85*, 648, (1993)).

This invention provides methods for inhibiting environmental estrogen effects comprising administering to a human or other animal in need thereof an effective amount of a compound of formula I



(I)

wherein R^1 and R^3 are independently hydrogen, $-CH_3$, $-C-(C_1-C_6\ alkyl)$, or $-C-Ar$, wherein Ar is optionally substituted phenyl;

R^2 is selected from the group consisting of pyrrolidino, hexamethyleneimino, and piperidino; and pharmaceutically acceptable salts and solvates thereof.

The current invention concerns the discovery that a select group of 2-phenyl-3-aroylbenzothiophenes (benzothiophenes), those of formula I, are useful for inhibiting environmental estrogen effects. In particular, the methods of the invention encompass inhibiting the reproductive effects of environmental estrogens.

The methods of use provided by this invention are practiced by administering to a human or other animal in need thereof a dose of a compound of formula I or a pharmaceutically acceptable salt or solvate thereof, that is effective to inhibit the effects of environmental estrogens. The term "inhibit" includes its generally accepted meaning

which includes prohibiting, preventing, restraining, and slowing, stopping or reversing. As such, the present method includes both medical therapeutic and/or prophylactic administration, as appropriate.

Raloxifene, a compound of this invention wherein it is the hydrochloride salt of a compound of formula 1, R^1 and R^3 are hydrogen and R^2 is 1-piperidinyl, is a nuclear regulatory molecule. Raloxifene has been shown to bind to the estrogen receptor and was originally thought to be a molecule whose function and pharmacology was that of an anti-estrogen in that it blocked the ability of estrogen to activate uterine tissue and estrogen dependent breast cancers. Indeed, raloxifene does block the action of estrogen in some cells; however in other cell types, raloxifene activates the same genes as estrogen does and displays the same pharmacology, e.g., osteoporosis, hyperlipidemia. As a result, raloxifene has been referred to as an anti-estrogen with mixed agonist-antagonist properties. The unique profile which raloxifene displays and differs from that of estrogen is now thought to be due to the unique activation and/or suppression of various gene functions by the raloxifene-estrogen receptor complex as opposed to the activation and/or suppression of genes by the estrogen-estrogen receptor complex. Therefore, although raloxifene and estrogen utilize and compete for the same receptor, the pharmacological outcome from gene regulation of the two is not easily predicted and is unique to each.

Generally, the compound is formulated with common excipients, diluents or carriers, and compressed into tablets, or formulated as elixirs or solutions for convenient oral administration, or administered by the intramuscular or intravenous routes. The compounds can be administered transdermally, and may be formulated as sustained release dosage forms and the like.

The compounds used in the methods of the current invention can be made according to established procedures, such as those detailed in U.S. Patent Nos. 4,133,814, 4,418,068, and 4,380,635 all of which are incorporated by reference herein. In general, the process starts with a benzo[b]thiophene having a 6-hydroxyl group and a 2-(4-hydroxyphenyl) group. The starting compound is protected, acylated, and deprotected to form the formula I compounds. Examples of the preparation of such compounds are provided in the U.S. patents discussed above. Optionally substituted phenyl includes phenyl and phenyl substituted once or twice with C_1-C_6 alkyl, C_1-C_4 alkoxy, hydroxy, nitro, chloro, fluoro, or tri(chloro or fluoro)methyl.

The compounds used in the methods of this invention form pharmaceutically acceptable acid and base addition salts with a wide variety of organic and inorganic acids and bases and include the physiologically acceptable salts which are often used in pharmaceutical chemistry. Such salts are also part of this invention. Typical inorganic acids used to form such salts include hydrochloric, hydrobromic, hydroiodic, nitric, sulfuric, phosphoric, hypophosphoric and the like. Salts derived from organic acids, such as aliphatic mono and dicarboxylic acids, phenyl substituted alkanoic acids, hydroxyalkanoic and hydroxyalkandioic acids, aromatic acids, aliphatic and aromatic sulfonic acids, may also be used. Such pharmaceutically acceptable salts thus include acetate, phenylacetate, trifluoroacetate, acrylate, ascorbate, benzoate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, bromide, isobutyrate, phenylbutyrate, ß-hydroxybutyrate, butyne-1,4-dioate, hexyne-1,4-dioate, caprate, caprylate, chloride, cinnamate, citrate, formate, fumarate, glycollate, heptanoate, hippurate, lactate, malate, maleate, hydroxymaleate, malonate, mandelate, mesylate,

nicotinate, isonicotinate, nitrate, oxalate, phthalate, teraphthalate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, propiolate, propionate, phenylpropionate, salicylate, sebacate, succinate, suberate, sulfate, bisulfate, pyrosulfate, sulfite, bisulfite, sulfonate, benzene-sulfonate, p-bromophenylsulfonate, chlorobenzenesulfonate, ethanesulfonate, 2-hydroxyethanesulfonate, methanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, p-toluenesulfonate, xylenesulfonate, tartarate, and the like. A preferred salt is the hydrochloride salt.

The pharmaceutically acceptable acid addition salts are typically formed by reacting a compound of formula I with an equimolar or excess amount of acid. The reactants are generally combined in a mutual solvent such as diethyl ether or benzene. The salt normally precipitates out of solution within about one hour to 10 days and can be isolated by filtration or the solvent can be stripped off by conventional means.

Bases commonly used for formation of salts include ammonium hydroxide and alkali and alkaline earth metal hydroxides, carbonates, as well as aliphatic and primary, secondary and tertiary amines, aliphatic diamines. Bases especially useful in the preparation of addition salts include ammonium hydroxide, potassium carbonate, methylamine, diethylamine, ethylene diamine and cyclohexylamine.

The pharmaceutically acceptable salts generally have enhanced solubility characteristics compared to the compound from which they are derived, and thus are often more amenable to formulation as liquids or emulsions.

Pharmaceutical formulations can be prepared by procedures known in the art. For example, the compounds can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and

the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following: fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

The compounds can also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes. Additionally, the compounds are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal tract, possibly over a period of time. The coatings, envelopes, and protective matrices may be made, for example, from polymeric substances or waxes.

The particular dosage of a compound of formula I required to inhibit the effects of environmental estrogens according to this invention will depend upon the severity of the condition, the route of administration, and related factors that will be decided by the attending physician. Generally, accepted and effective daily doses will be from about 0.1 to about 1000 mg/day, and more typically from about 50 to about 200 mg/day. Such dosages will be administered to a subject in need thereof from once to about three times

each day, or more often as needed to effectively treat the syndrome or at least one of its symptoms.

It is usually preferred to administer a compound of formula I in the form of an acid addition salt, as is customary in the administration of pharmaceuticals bearing a basic group, such as the piperidino ring. It is also advantageous to administer such a compound by the oral route. For such purposes the following oral dosage forms are available.

In the formulations which follow, "Active ingredient" means a compound of formula I.

Formulation 1: Gelatin Capsules

Hard gelatin capsules are prepared using the following:

Ingredient	Quantity (mg/capsule)
Active ingredient	0.1 - 1000
Starch, NF	0 - 650
Starch flowable powder	0 - 650
Silicone fluid 350 centistokes	0 - 15

The ingredients are blended, passed through a No. 45 mesh U.S. sieve, and filled into hard gelatin capsules.

Examples of specific capsule formulations of raloxifene that have been made include those shown below:

Formulation 2: Raloxifene capsule

Ingredient	Quantity (mg/capsule)
Raloxifene	1
Starch, NF	112
Starch flowable powder	225.3
Silicone fluid 350 centistokes	1.7

Formulation 3: Raloxifene capsule

Ingredient	Quantity (mg/capsule)
Raloxifene	5
Starch, NF	108
Starch flowable powder	225.3
Silicone fluid 350 centistokes	1.7

Formulation 4: Raloxifene capsule

Ingredient	Quantity (mg/capsule)
Raloxifene	10
Starch, NF	103
Starch flowable powder	225.3
Silicone fluid 350 centistokes	1.7

Formulation 5: Raloxifene capsule

Ingredient	Quantity (mg/capsule)
Raloxifene	50
Starch, NF	150
Starch flowable powder	397
Silicone fluid 350 centistokes	3.0

The specific formulations above may be changed in compliance with the reasonable variations provided.

A tablet formulation is prepared using the ingredients below:

Formulation 6: Tablets

Ingredient	Quantity (mg/tablet)
Active ingredient	0.1 - 1000
Cellulose, microcrystalline	0 - 650
Silicon dioxide, fumed	0 - 650
Stearate acid	0 - 15

The components are blended and compressed to form tablets.

Alternatively, tablets each containing 0.1 - 1000 mg of active ingredient are made up as follows:

Formulation 7: Tablets

Ingredient	Quantity (mg/tablet)
Active ingredient	0.1 - 1000
Starch	45
Cellulose, microcrystalline	35
Polyvinylpyrrolidone (as 10% solution in water)	4
Sodium carboxymethyl cellulose	4.5
Magnesium stearate	0.5
Talc	1

The active ingredient, starch, and cellulose are passed through a No. 45 mesh U.S. sieve and mixed thoroughly. The solution of polyvinylpyrrolidone is mixed with the resultant powders which are then passed through a No. 14 mesh U.S. sieve. The granules so produced are dried at 50°-60° C and passed through a No. 18 mesh U.S. sieve. The sodium carboxymethyl starch, magnesium stearate, and talc, previously passed through a No. 60 U.S. sieve, are then added

to the granules which, after mixing, are compressed on a tablet machine to yield tablets.

Suspensions each containing 0.1 - 1000 mg of medicament per 5 mL dose are made as follows:

Formulation 8: Suspensions

Ingredient	Quantity (mg/5 ml)
Active ingredient	0.1 - 1000 mg
Sodium carboxymethyl cellulose	50 mg
Syrup	1.25 mg
Benzoic acid solution	0.10 mL
Flavor	q.v.
Color	q.v.
Purified water to	5 mL

The medicament is passed through a No. 45 mesh U.S. sieve and mixed with the sodium carboxymethyl cellulose and syrup to form a smooth paste. The benzoic acid solution, flavor, and color are diluted with some of the water and added, with stirring. Sufficient water is then added to produce the required volume.

Test Procedure

Assay 1

In the examples illustrating the methods, a post-menopausal model was used in which effects of different treatments upon circulating lipids were determined.

Seventy-five day old female Sprague Dawley rats (weight range of 200 to 225g) were obtained from Charles River Laboratories (Portage, MI). The animals were bilaterally ovariectomized (OVX) Charles River Laboratories, and shipped after one week. Upon arrival, they were housed in metal hanging cages in groups of 3 or 4 per cage and had *ad libitum* access to food (calcium content approximately

0.5%) and water for one week. Room temperature was maintained at 22.2° ± 1.7°C with a minimum relative humidity of 40%. The photoperiod in the room was 12 hours light and 12 hours dark.

Dosing Regimen Tissue Collection. After a one week acclimation period (therefore, two weeks post-OVX) daily dosing with test compound raloxifene was initiated. Zearanol and the test compound were given orally in a 20% cyclodextrin vehicle. Animals were dosed daily for 4 days. Following the dosing regimen, animals were weighed and anesthetized with a ketamine: Xylazine (2:1, V:V) mixture and a blood sample was collected by cardiac puncture. The animals were then sacrificed by asphyxiation with CO_2, the uterus was removed through a midline incision, and a wet uterine weight was determined.

Cholesterol Analysis. Blood samples were allowed to clot at room temperature for 2 hours, and serum was obtained following centrifugation for 10 minutes at 3000 rpm. Serum cholesterol was determined using a Boehringer Mannheim Diagnostics high performance cholesterol assay. Briefly the cholesterol was oxidized to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide was then reacted with phenol and 4-aminophenazone in the presence of peroxidase to produce a p-quinone imine dye, which was read spectrophotemetrically at 500 nm. Cholesterol concentration was then calculated against a standard curve. The entire assay was automated using a Biomek Automated Workstation.

Uterine Eosinophil Peroxidase (EPO) Assay. Uteri were kept at 4°C until time of enzymatic analysis. The uteri were then homogenized in 50 volumes of 50 mM Tris buffer (pH - 8.0) containing 0.005% Triton X-100. Upon addition of 0.01% hydrogen peroxide and 10 mM o-phenylenediamine (final concentrations) in Tris buffer, increase in absorbance was monitored for one minute at 450 nm. The presence of

eosonophils in the uterus is an indication of estrogenic activity of a compound. The maximal velocity of a 15 second interval was determined over the initial, linear portion of the reaction curve.

Data Summary. As demonstrated in Table 1, the example environmental estrogen, zearanol, produced a three-fold increase in uterine wet weight and led to a significant increase in uterine eosinophilia (EPO Vmax for OVX controls was 4 mOD/min) and a reduction in serum cholesterol. Each of these activities are qualitatively similar to those produced by estrogenic drugs (i.e. 17α ethynyl-estradiol) in this assay system.

The test compound when given in combination with zearanol, bllunted the uterine stimulatory and cholesterol reducing activity of the environmental estrogen. At dose levels of 10 mg/kg, both test compounds led to significant attenuation of zearanol induced uterine weight stimulation, uterine EPO elevation and serum cholesterol reduction.

TABLE 1

	Uterine Wt. (% ↑ vs OVX control)	Uterine EPO (Vmax)	Serum Cholesterol (% ↓ vs. OVX control)
Zearanol (10mg/kg)	200.5	403.8	82.8
Zearanol 10mg/kg + Test Cmpd. (0.1 mg/kg)	189.2	429.6	77.7
Zearanol 10mg/kg + Test Cmpd. (1 mg/kg)	202.2	360.3	66.2
Zearanol 10mg/kg + Test Cmpd. (10 mg/kg)	91.2	177.9	52.4
17 α-ethynylestradiol (0.1 mg/kg)	109.2	177.9	86.4

Assay 2

Between 5 and 40 individuals with known exposure to an environmental estrogen who present symptomatology (i.e. cancer, gynocomostia, decreased sperm count) are used. The individuals are treated with a compound of formula I for between four and twelve months, during which time the effects of the environmental estrogen are measured for regression.

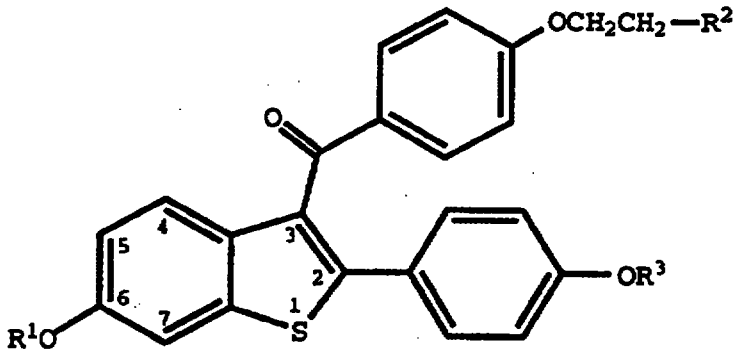
Utility of the compounds of the invention is illustrated by the impact they have on the effects of environmental estrogens when used in one of the studies above.

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We claim:

1. A method of inhibiting the effects of environmental estrogens comprising administering to a human or other animal in need thereof an effective amount of a compound having the formula



(I)

wherein R^1 and R^3 are independently hydrogen, $-CH_3$, $-\overset{O}{\overset{\|}{C}}-(C_1-C_6\ \text{alkyl})$, or $-\overset{O}{\overset{\|}{C}}-Ar$, wherein Ar is optionally substituted phenyl;

R^2 is selected from the group consisting of pyrrolidine, hexamethyleneimino, and piperidino; or a pharmaceutically acceptable salt of solvate thereof.

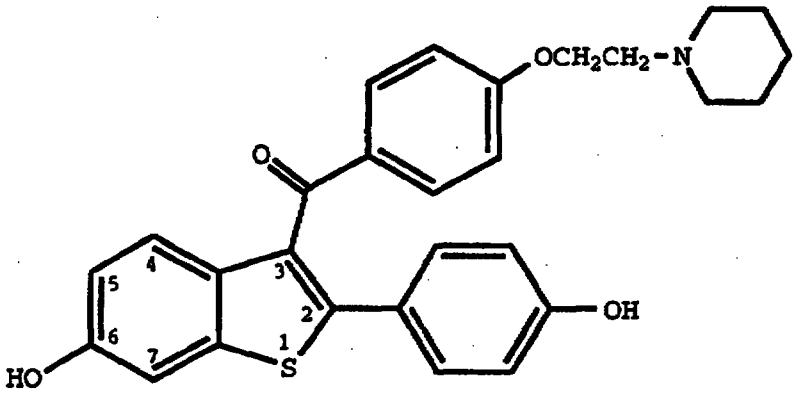
2. The method of Claim 1 wherein said compound is the hydrochloride salt thereof.

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3. The method of Claim 1 wherein said compound is



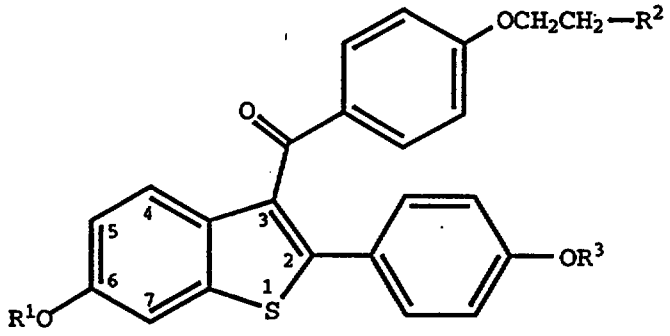
or its hydrochloride salt.

4. The method of Claim 1 wherein said effects involve the reproductive system.

* C L M S *

We claim:

1. A use of an effective amount of a compound having the formula



(I)

wherein R^1 and R^3 are independently hydrogen, $-CH_3$, $-\overset{O}{\overset{\|}{C}}-(C_1-C_6\ \text{alkyl})$, or $-\overset{O}{\overset{\|}{C}}-Ar$, wherein Ar is optionally substituted phenyl;

R^2 is selected from the group consisting of pyrrolidine, hexamethyleneimino, and piperidino; or a pharmaceutically acceptable salt of solvate thereof in a preparation of a medicament for inhibiting the effects of environmental estrogens in a subject in need thereof.

2. The use of Claim 1 wherein said compound is the hydrochloride salt thereof.

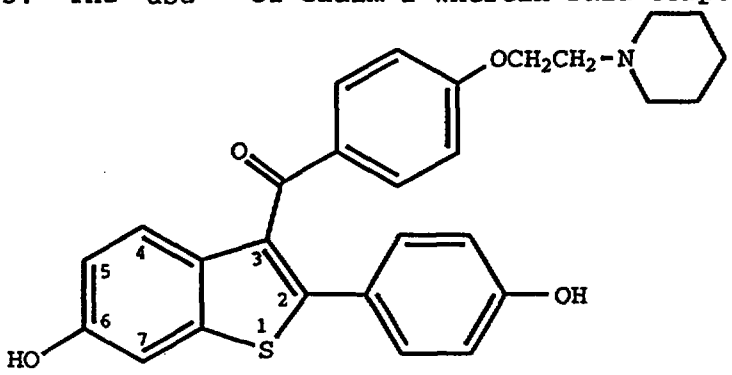
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3. The use of Claim 1 wherein said compound is



or its hydrochloride salt.

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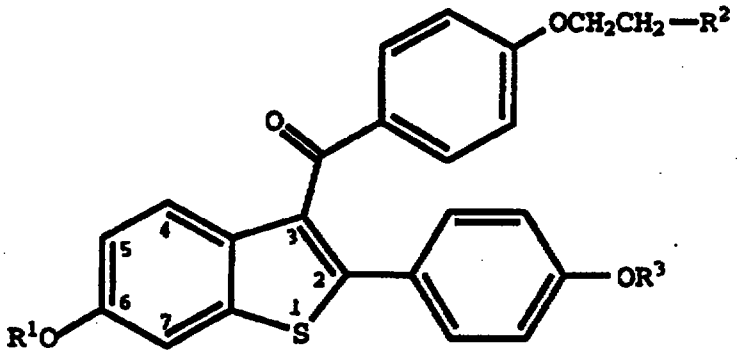
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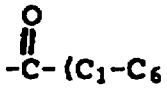
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(I)



(a)



(b)

(57) Abstract

A method of inhibiting effects of environmental estrogens comprising administering to a human or other animal in need thereof an effective amount of a compound having formula (I), wherein R^1 and R^3 are independently hydrogen, $-CH_3$, (a) or (b), wherein Ar is optionally substituted phenyl; R^2 is selected from the group consisting of pyrrolidine, hexamethyleneimino, and piperidino; or a pharmaceutically acceptable salt of solvate thereof.

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