(12) UK Patent Application (19) GB (11) 2 363 984 (13) A

(43) Date of A Publication 16.01.2002

(21) Application No 0016027.5

(22) Date of Filing 29.06.2000

(71) Applicant(s)
Hunter-Fleming Limited
(Incorporated in the United Kingdom)
8 Centre One, Lysander Way, Old Sarum Park,
SALISBURY, Wiltshire, SP4 6BU, United Kingdom

(72) Inventor(s)
Ernst Wulfert
Ashley Ker Pringle
Lars Eric Sundstrom

(74) Agent and/or Address for Service
Marks & Clerk
57-60 Lincoln's Inn Fields, LONDON, WC2A 3LS,
United Kingdom

(51) INT CL^7
A61K 31/56 31/565 31/566 31/5685 31/57 , A61P 25/28

(52) UK CL (Edition T)
A5B BHA B180 B24Y B240 B244
U1S S1325 S2416

(56) Documents Cited
GB 2317826 A **WO 97/37664 A2**
WO 94/20111 A1 **WO 94/03176 A1**
US 5763433 A
Immunol. Cell Biol. Vol. 78, No. 3, 2000, pages 238-246

(58) Field of Search
UK CL (Edition R) **A5B**
INT CL^7 **A61K 31/565 31/566 31/5685 31/57**
Online: PAJ, EPODOC, WPI, TXTE, CAS-ONLINE

(54) Abstract Title
Protection against neuronal damage using 3-hydroxy-7β-hydroxy steroids and 3-oxo-7β-hydroxy steroids

(57) 3-Hydroxy-7β-hydroxy steroids and 3-oxo-7β-hydroxy steroids and pharmaceutically acceptable esters thereof are used for protection against neuronal damage. Such steroids include 7β-hydroxyepiandrosterone, 7β-hydroxydehydroepiandrosterone, 7β-hydroxy-17β-oestradiol, 7β-hydroxypregnenolone and 7β-hydroxyoestrone. The neuronal damage may be that caused by Alzheimer's Disease, Parkinson's Disease, Cognitive Impairment No Dementia, stroke, brain trauma, spinal cord injury or peripheral nerve injury.

GB 2 363 984 A

NEUROPROTECTIVE STEROIDS

The present invention relates to the use of a series of 3-hydroxy-7β-hydroxy-steroid compounds and certain ketone derivatives thereof for protection against neuronal cell death, and which are thus useful in the treatment and prevention of such conditions or the sequelae of such conditions as Alzheimer's Disease, Parkinson's Disease, Cognitive Impairment No Dementia (CIND), stroke, brain trauma, spinal cord injury and peripheral nerve injury; they are also useful for enhancing cognitive function.

The production of 7α-hydroxylated metabolites of dehydroepiandrosterone (DHEA) *in vivo* has been known since 1959 with the identification of 7α-hydroxy-DHEA in urine [J J Schneider, M L Lewbart, Recent Progr. Horm. Res. 15 (1959) 201-230; L Starka *et al*, Clin. Chim. Acta. 7 (1961) 309-316)]. Since then, extensive 7α-hydroxylation of 3β-hydroxysteroid substrates (including DHEA and epiandrosterone – EPIA) has been reported in tissue preparations from many human organs, including adult and foetal liver, testes, epididymus, skin, mammary tissue, prostate, adipose stromal cells and tonsils. Hydroxylation of DHEA at the 7-position has also been demonstrated in rat liver and in numerous mouse tissues and organs. In all these studies, 7α-hydroxy-DHEA was by far the major metabolite produced. Indeed, Doostzadeh *et al* [Steroids 63 (1998) 608-614] reported that the production rate of 7α-hydroxy-DHEA by mouse liver microsomes was more than fifteen times the production rate of 7β-hydroxy-DHEA.

EPIA, DHEA and pregnenolone have also been shown to be rapidly and extensively transformed to their corresponding 7α-hydroxy metabolites in the rat brain [J M Guiraud *et al*, Steroids 34 (1979) 241-248; M Warner *et al*, Endocrinology 124 (1989) 2699-2706; Y Akwa *et al*, Biochem. J. 288 (1992) 959-964)].

WO97/37664 discloses the use of a variety of compounds, including 7α-hydroxy-substituted steroids, to treat neuropsychiatric, immune or endocrine disorders. Among the disorders suggested in WO97/37664 that these compounds may be used to treat is included Alzheimer's Disease. However, the mechanism suggested for this action is that the disorder is hypothesised to result from a deficit of the 7α-

hydroxy-substituted steroid in the brain, and the treatment proposed in WO97/37664 thus rectifies this deficit by the administration of a 7α-hydroxy-substituted steroid to replace the missing compound. The procedure described in WO97/37664 thus treats an existing condition, rather than preventing the condition or preventing a worsening of the condition by preventing further neuronal damage. WO97/37664 does not, therefore, describe a neuroprotective effect. It is also predicated upon the belief that the active agent is the 7α compound, and that the 7β compound, if present, is inactive.

Although the 7β-hydroxy analogues of the compounds disclosed in WO97/37664 were known to be produced *in vivo*, they are produced in amounts of less than 5%, as compared with more than 95% of the 7α-isomer. For this reason and in the light of the research summarised above, it is clear from the literature that the general expectation was that the 7β-isomer would be inactive. As a result, as is clear from the literature summarised above, and a great deal more, that essentially no investigation of possible biological activity of the 7β compounds has been carried out.

Contrary to this expectation, we have surprisingly found that the 7β-hydroxy-substituted steroids do have a biological activity, and that this activity is not the activity as described in WO97/37664 for the 7α-hydroxy-substituted steroids. Rather it is a neuroprotective activity such as has previously been demonstrated, albeit in a different class of compounds, in WO99/31049.

In events such as prolonged hypoxia and ischaemia, which may or may not be associated with hypoglycaemia, neuronal damage, to varying degrees, is encountered.

Ischaemia typically occurs during heart attacks, but the damage incurred at these times is substantially limited to the heart tissues, and certain treatments have been developed. With regard to the present invention, we are concerned the effects of both short term and more long term ischaemia on the brain, such as occurs with stroke patients or as a result of head injury, and also in more slowly developing neurodegenerative diseases in ageing where chronic sub-threshold levels of ischaemia and/or compromised energy supply may contribute to the brain degenerative changes observed. The severity of the ischaemia depends on the nature of the stroke or injury, but, invariably, there is brain damage, and it is this which the present invention

addresses.

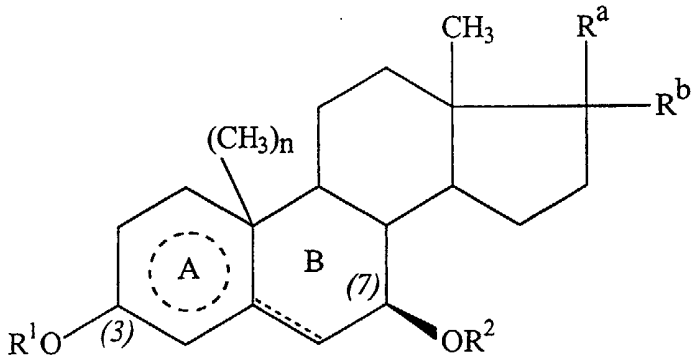
Various neuroprotective agents are known in the art which attempt to alleviate the problem of brain damage, but all of those currently known tend to be associated with adverse side effects. For example, MK801 (dizocilpine maleate) is a fairly simple molecule and is known to provide a level of neuroprotection to ischaemic patients. However, MK801 is also associated with "alarming psychotropic effects" (Martindale), as well as adverse motor effects. The neuroprotective effects are detailed in Brain Research 755 (1997) 36-46 (Pringle, A.K., *et al*), incorporated herein by reference. These same authors also described the neuroprotective effects of conotoxin in an earlier paper but, despite the neuroprotective effects of this compound, adverse side effects, *in vivo*, are observed.

Thus, the present invention consists in the use for the manufacture of a medicament for protection against neuronal damage of a 3-hydroxy-7β-hydroxy steroid or a 3-oxo-7β-hydroxy steroid and pharmaceutically acceptable esters thereof.

A particular class of 7β-hydroxy steroids which are of especial interest to the present invention are the 3β,7β-dihydroxy steroids and pharmaceutically acceptable esters thereof.

Preferred esters are carboxylic acid esters.

Examples of optionally substituted 3β,7β-dihydroxy steroids and pharmaceutically acceptable esters and other derivatives thereof which may be used in the present invention are those compounds of formula (I):



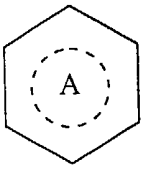
(I)

wherein

R^1 and R^2 are the same as or different from each other and each represents a hydrogen atom, an alkyl group having from 1 to 6 carbon atoms, an alkenyl group having from 2 to 6 carbon atoms, an alkynyl group having from 2 to 6 carbon atoms, an aryl group having from 6 to 10 carbon atoms, a formyl group, an alkylcarbonyl group having from 2 to 7 carbon atoms, an alkenylcarbonyl group having from 3 to 7 carbon atoms, an alkynylcarbonyl group having from 3 to 7 carbon atoms, an arylcarbonyl group having from 7 to 11 carbon atoms, an aralkylcarbonyl group having from 8 to 15 carbon atoms, an aralkenylcarbonyl group having from 9 to 15 carbon atoms, or a heterocyclic-carbonyl group, as defined below;

one of R^a and R^b represents a group of formula $-R^c$, preferably in the β configuration, and the other represents a hydrogen atom, or R^a and R^b together represent an oxo group;

R^c represents an alkanoyl group having from 1 to 6 carbon atoms, an aryl-carbonyl group, in which the aryl part is an aromatic carbocyclic group having from 6 to 10 ring carbon atoms, a heterocyclic-carbonyl group, as defined below, or a group of formula $-OR^4$, where R^4 represents any one of the groups and atoms defined above for R^1 and R^2;

the ring A, 

, is a benzene or cyclohexane ring;

when ring A is a cyclohexane ring, the dotted line in ring B represents a single or double carbon-carbon bond and n is 1; or when ring A is a benzene ring, the dotted line in ring B represents a single carbon-carbon bond and n is 0;

said heterocyclic-carbonyl group is a group of formula R^3–CO , where R^3 represents a heterocyclic group having from 3 to 7 ring atoms, of which from 1 to 3 are hetero-atoms selected from nitrogen atoms, oxygen atoms and sulphur atoms, and the

remaining atom or atoms of which there is at least one is or are carbon atoms;

said alkyl, alkenyl and alkynyl groups and the alkyl, alkenyl and alkynyl parts of said alkylcarbonyl, alkenylcarbonyl and alkynylcarbonyl groups being unsubstituted or having at least one of the following substituents ψ:

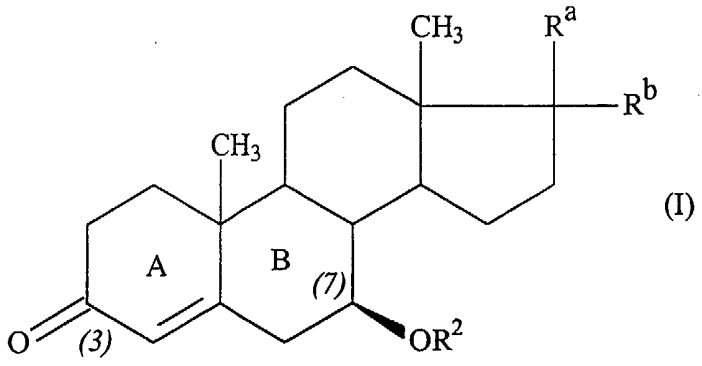
substituents ψ: hydroxy groups, mercapto groups, halogen atoms, amino groups, alkylamino groups having from 1 to 6 carbon atoms, dialkylamino groups in which each alkyl group has from 1 to 6 carbon atoms, carbamoyl groups, nitro groups, alkoxy groups having from 1 to 6 carbon atoms, alkylthio groups having from 1 to 6 carbon atoms, carboxy groups, alkoxycarbonyl groups and unsubstituted aryl groups having from 6 to 10 carbon atoms;

said aryl groups, said heterocyclic groups, and the aryl parts of said arylcarbonyl groups and said aralkylcarbonyl groups being unsubstituted or having at least one of the following substituents β:

substituents ξ: any of substituents ψ, and alkyl groups having from 1 to 6 carbon atoms, hydroxyalkyl groups having from 1 to 6 carbon atoms, and haloalkyl groups having from 1 to 6 carbon atoms;

and pharmaceutically acceptable salts and esters thereof.

Examples of 3-oxo-7β-hydroxy steroids which may be used in the present invention are those compounds of formula (II):



(I)

in which R^a, R^b and R^2 are as defined above.

In the compounds of the present invention, where R^1, R^2, R^4 or substituent ξ is an alkyl group, this may be a straight or branched chain alkyl group having from 1 to 6 carbon atoms, and examples include the methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, t-butyl, pentyl, 1-methylbutyl, 2-methylbutyl, 3-methylbutyl, 1-ethylpropyl, 2-ethylpropyl, 1,1-dimethylpropyl, hexyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, 1-ethylbutyl, 2-ethylbutyl, 3-ethylbutyl, t-hexyl, and 1,1-dimethylpentyl groups, of which those groups having from 1 to 4 carbon atoms are preferred, the methyl and ethyl groups being most preferred.

Where R^1, R^2 or R^4 represents an alkenyl group, this may be a straight or branched chain alkenyl group having from 2 to 6 carbon atoms, and examples include the vinyl, 1-propenyl, allyl, isopropenyl, methallyl, 1-, 2-, 3-butenyl, isobutenyl, 1-, 2-, 3-, 4-pentenyl and 1-, 2-, 3-, 4-, 5-hexenyl groups, of which those alkenyl groups having from 2 to 4 carbon atoms are preferred, the vinyl and allyl groups being most preferred.

Where R^1, R^2 or R^4 represents an alkynyl group, this may be a straight or branched chain alkynyl group having from 2 to 6 carbon atoms, and examples include the ethynyl, 1-, 2-propynyl, 1-, 2-, 3-butynyl, isobutynyl, 1-, 2-, 3-, 4-pentynyl and 1-, 2-, 3-, 4-, 5-hexynyl groups, of which those alkynyl groups having from 2 to 4 carbon atoms are preferred.

Where R^1, R^2, R^4 or substituent ψ represents an aryl group, this is an aromatic carbocyclic group having from 6 to 10 carbon atoms. Examples of such groups include the phenyl, 1-naphthyl, 2-naphthyl and indenyl groups, of which the phenyl group is preferred. Except in the case of substituent α, these groups may be substituted or unsubstituted. Where the group is substituted, the number of substituents is limited only by the number of substitutable positions, and possibly, in some instances, by steric constraints. Thus, in the case of the phenyl groups, the maximum number of substituents is 5, in the case of the naphthyl groups, the maximum number of substituents is 7 and so on. However, a preferred number of substituents is from 1 to 3, and the substituents are as hereafter described.

Where R^1, R^2 or R^4 represents an alkylcarbonyl group, this is an alkanoyl group, which may be a straight or branched chain group having from 2 to 7 carbon atoms (i.e. from 1 to 6 carbon atoms in the alkyl part), and examples include the acetyl, propionyl, butyryl, isobutyryl, valeryl, isovaleryl, pivaloyl, hexanoyl, and heptanoyl groups, of which those groups having from 2 to 5 carbon atoms are preferred, the acetyl and propionyl groups being most preferred. The alkyl portion of this group may be substituted or unsubstituted, and, if substituted, the substituents are selected from substituents α. Examples of such substituted groups include the alanyl, β-alanyl, phenylalanyl, asparaginyl, cysteinyl, glycoloyl, glycyl, methionyl, ornithyl, glyceroyl, tropoyl, glutaminyl, glutamyl, homocysteinyl, seryl, homoseryl, threonyl, lactoyl, leucyl, isoleucyl, norleucyl, lysyl, valyl, norvalyl and sarcosyl groups.

Where R^1, R^2 or R^4 represents an alkenylcarbonyl group, this may be a straight or branched chain alkenylcarbonyl group having from 3 to 7 carbon atoms, and examples include the acryloyl, methacryloyl, crotonoyl, isocrotonoyl, 3-butenoyl, pentenoyl and hexenoyl groups, of which those alkenylcarbonyl groups having from 3 to 5 carbon atoms are preferred, the acryloyl and methacryloyl groups being most preferred.

Where R^1, R^2 or R^4 represents an alkynylcarbonyl group, this may be a straight or branched chain alkynylcarbonyl group having from 3 to 7 carbon atoms, and examples include the propioloyl, 3-butynylcarbonyl, pentynylcarbonyl and hexynylcarbonyl groups, of which those alkynylcarbonyl groups having from 3 to 5 carbon atoms are preferred.

Where R^c, R^1, R^2 or R^4 represents an arylcarbonyl group, the aryl part of this may be any of the aryl groups defined and exemplified above. Preferred arylcarbonyl groups include the benzoyl, o-, m- or p-toluoyl, o-, m- or p-anisoyl, o-, m- or p-hydroxybenzoyl, picryl, galloyl, protocatechuoyl, vanilloyl, veratroyl, anthraniloyl, 1-naphthoyl and 2-naphthoyl groups.

Where R^1, R^2 or R^4 represents an aralkylcarbonyl or aralkenylcarbonyl group, the aryl and, as the case may be, alkyl or alkenyl group may be any of those groups

defined and exemplified above. Specific examples of such groups include the phenylacetyl, 3-phenylpropionyl, benziloyl, tyrosyl, atropoyl, hydratropoyl and cinnamoyl groups.

Where R^c, R^1, R^2 or R^4 represents a heterocyclic-carbonyl group, this is a group of formula R^3–CO-, where R^3 represents a heterocyclic group having from 3 to 7 ring atoms, of which from 1 to 3 are nitrogen, oxygen or sulphur atoms, the remainder being carbon atoms. At least one of the ring atoms should be a carbon atom.. Where there are 3 hetero-atoms, it is preferred that at least one is a nitrogen atom. Examples of such groups include the 2- and 3-furoyl, 2- and 3-thenoyl, 2-pyridinecarbonyl, nicotinoyl, isonicotinoyl, prolyl, piperidinecarbonyl, piperazinecarbonyl and morpholinocarbonyl groups.

Where R^c represents an alkanoyl group, this may be a straight or branched chain group having from 1 to 6 carbon atoms, and examples include the formyl, acetyl, propionyl, butyryl, isobutyryl, valeryl, isovaleryl, pivaloyl, hexanoyl, and heptanoyl groups, of which those groups having from 2 to 5 carbon atoms are preferred, the acetyl and propionyl groups being more preferred, and the acetyl group being most preferred.

Where substituent ψ or substituent ξ is an alkylamino group having from 1 to 6 carbon atoms, the alkyl part may be any of the alkyl groups defined and exemplified above. Preferred examples of such alkylamino groups include the methylamino, ethylamino, propylamino, isopropylamino, butylamino, isobutylamino, sec-butylamino, t-butylamino, pentylamino, isopentylamino, neopentylamino, t-pentylamino, hexylamino, and isohexylamino groups, of which those groups having from 1 to 4 carbon atoms are preferred, the methylamino and ethylamino groups being most preferred.

Where substituent ψ or substituent ψ is a dialkylamino group, each alkyl part has from 1 to 6 carbon atoms, and the two alkyl groups may be the same as or different from each other. The alkyl groups may be any of the alkyl groups defined and exemplified above. Preferred examples of such dialkylamino groups include the

dimethylamino, methylethylamino, diethylamino, methylpropylamino, dipropylamino, diisopropylamino, ethylbutylamino, dibutylamino, di-t-butylamino, methylpentylamino, dipentylamino, diisopentylamino, and dihexylamino groups, of which those groups having from 1 to 4 carbon atoms in each alkyl group are preferred, the dimethylamino and diethylamino groups being most preferred.

Where substituent ψ or substituent ξ is an alkoxy group, this may be a straight or branched chain alkoxy group having from 1 to 6 carbon atoms, and examples include the methoxy, ethoxy, propoxy, isopropoxy, butoxy, isobutoxy, sec-butoxy, t-butoxy, pentyloxy, isopentyloxy, neopentyloxy, t-pentyloxy, hexyloxy, and isohexyloxy groups, of which those groups having from 1 to 4 carbon atoms are preferred, the methoxy and ethoxy groups being most preferred.

Where substituent ψ or substituent ζ is an alkylthio group having from 1 to 6 carbon atoms, the alkyl part may be any of the alkyl groups defined and exemplified above. Preferred examples of such alkylthio groups include the methylthio, ethylthio, propylthio, isopropylthio, butylthio, isobutylthio, sec-butylthio, t-butylthio, pentylthio, isopentylthio, neopentylthio, t-pentylthio, hexylthio, and isohexylthio groups, of which those groups having from 1 to 4 carbon atoms are preferred, the methylthio and ethylthio groups being most preferred.

Where substituent ψ or substituent ξ is an alkoxycarbonyl group, this may be a straight or branched chain alkoxycarbonyl group having from 2 to 7 carbon atoms, and examples include the methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, isopropoxycarbonyl, butoxycarbonyl, isobutoxycarbonyl, sec-butoxycarbonyl, t-butoxycarbonyl, pentyloxycarbonyl, isopentyloxycarbonyl, neopentyloxycarbonyl, t-pentyloxycarbonyl, hexyloxycarbonyl, and isohexyloxycarbonyl groups, of which those groups having from 1 to 4 carbon atoms are preferred, the methoxycarbonyl and ethoxycarbonyl groups being most preferred.

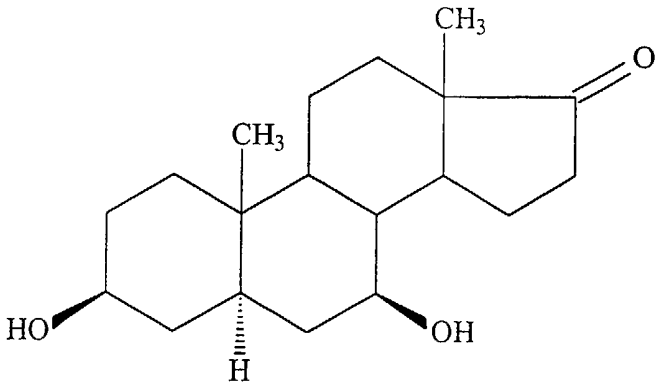
Where substituent ξ is a hydroxyalkyl group having from 1 to 6 carbon atoms, the alkyl part may be any of the alkyl groups defined and exemplified above. Preferred examples of such hydroxyalkyl groups include the hydroxymethyl, 1- and 2-hydroxyethyl, 1-, 2- and 3-hydroxypropyl, 1,2-dihydroxyethyl, 1,2,3-trihydroxy-

propyl, 4-hydroxybutyl, 5-hydroxypentyl and 6-hydroxyhexyl groups.

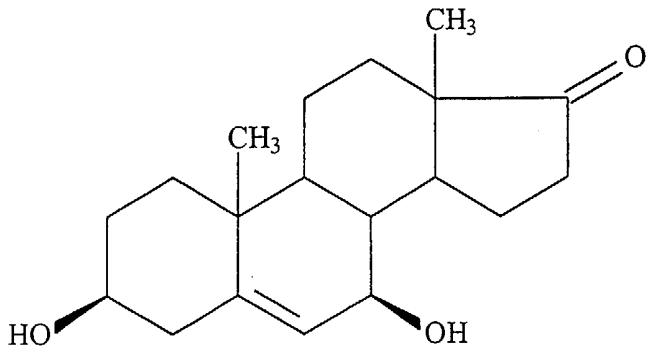
Where substituent ξ is a haloalkyl group having from 1 to 6, preferably from 1 to 4, carbon atoms, the alkyl part may be as defined and exemplified above, and the halogen atom is preferably chlorine, fluorine, bromine or iodine. Examples of such groups include the fluoromethyl, chloromethyl, bromomethyl, iodomethyl, dichloromethyl, difluoromethyl, trichloromethyl, trifluoromethyl, 2,2,2-trichloroethyl, 2-chloroethyl, 2-fluoroethyl, 2-bromoethyl, 2-iodoethyl, 2,2-dibromoethyl, 2,2,2-tribromoethyl, 3-fluoropropyl, 3-chloropropyl, 4-bromobutyl, 4-fluorobutyl, 5-fluoropentyl and 6-fluorohexyl groups.

It will be appreciated that, where the compound contains a group of formula –OR, where R is any of the groups and atoms defined above in relation to R^1 etc., the active species is likely to be the compound containing the free hydroxy group. Accordingly, any group that can be converted *in vivo* to a hydroxy group may be used in place of the hydroxy group.

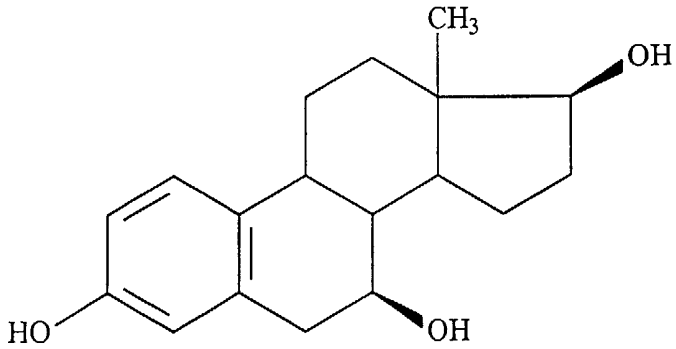
Specific examples of compounds of the present invention include:



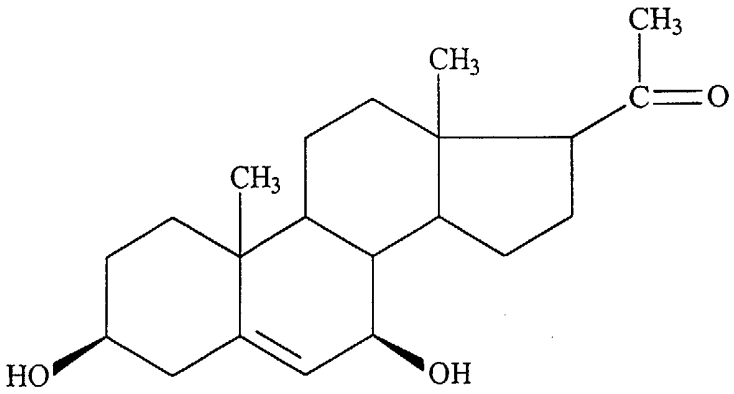
7β-hydroxy-epiandrosterone
(7β-hydroxy-EPIA)



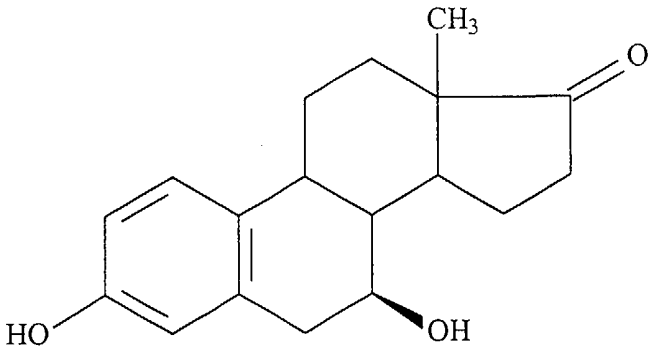
7β-hydroxy-dehydroepiandrosterone
(7β-hydroxy-DHEA)



7β-hydroxy-17β-oestradiol

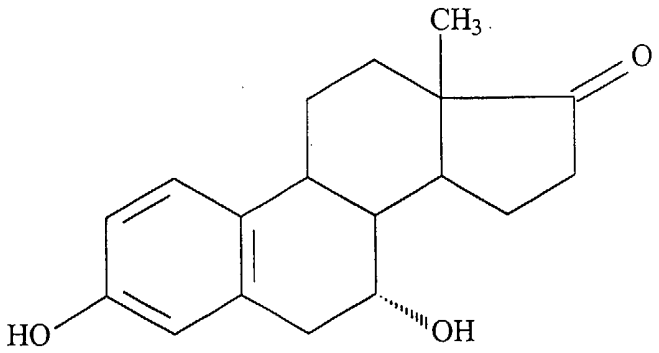


7β-hydroxy-pregnenolone



7β-hydroxy-oestrone

In addition, the following 7α-hydroxy compound is thought to be active in the same way:



7α-hydroxy-oestrone

We have surprisingly discovered that these compounds can be used to protect against acute and chronic neuronal damage caused by such events as stroke, brain trauma and cerebral ischaemia such as may be induced by sub-arachnoid haemhorrage or which occurs during heart bypass surgery etc.

The compounds of the present invention may be prepared by a variety of processes, well known in themselves, starting from the parent steroids. For example, they may be prepared by the methods described in the literature referred to above, which would give a mixture of the 7β and corresponding 7α compounds, which may then be separated by well known techniques.

As an example, 7β-hydroxy EPIA may be obtained from DHEA by allylic

oxidation after protection of the 3β-hydroxy group and the 17-ketone group using conventional methods. The product is then reduced with a soluble metal compound catalyst (such as sodium hydride) and the 3β-hydroxy and 17-ketone groups are deprotected. The 7α-hydroxy and 7β-hydroxy epimers may then be separated by conventional means, for example column chromatography, and the 7β-hydroxy EPIA may be crystallised to purity.

The compounds of the present invention may be applied to the patient if it is suspected that they are in danger of an ischaemic event, especially a stroke or head injury. Such prophylactic application may be exceedingly useful. However, it has also been demonstrated that the compounds of the present invention have useful activity, even if applied after an ischaemic event, but it will be appreciated that it is preferred to administer the compounds as soon as possible, in order to avoid as much neuronal degeneration as possible. In some circumstances it may be desirable to administer repeated doses, especially where the patient remains in danger of an ischaemic event.

Suitable methods of administration are generally by injection, in order to achieve the desired result as soon as possible. Thus, intravenous injection is particularly preferred but, in some circumstances it may be preferable to administer the compound directly into the cerebrospinal fluid.

The dose of the compound of the present invention will vary depending upon many factors, including the age, body weight and general condition of the patient, as well as the mode, frequency and route of administration. However, a dose of from 0.01 to 50 mg/kg body weight is generally recommended, a dose of from 0.05 to 20 mg/kg body weight being more preferred. This may be administered in a single dose or in divided doses.

Protocol For Studying Hypoxic Neuronal Damage

Organotypic hippocampal slice cultures were prepared using the basic method of Pringle *et al* (1996, 1997) modified as follows:

Wistar rat pups (8-11 days old) were decapitated and the hippocampus rapidly

dissected into ice-cold Gey's balanced salt solution supplemented with 4.5mg/ml glucose. Slices were separated and plated onto Millicell CM culture inserts (4 per well) and maintained at 37°C/5% CO_2 for 14 days. Maintenance medium consisted of 25% heat-inactivated horse serum, 25% Hank's balanced salt solution (HBSS) and 50% minimum essential medium with added Earle's salts (MEM) supplemented with 1mM glutamine and 4.5mg/ml glucose. Medium was changed every 3-4 days.

Experimental hypoxia was performed as described previously (Pringle *et al.*, 1996; 1997). Briefly, cultures were transferred to serum free medium (SFM – 75% MEM, 25% HBSS supplemented with 1mM glutamine and 4.5mg/ml glucose) containing 5μg/ml of the fluorescent exclusion dye propidium iodide (PI). Cultures were allowed to equilibrate in SFM for 60 minutes prior to imaging. PI fluorescence was detected using a Leica inverted microscope fitted with a rhodamine filter set. Any cultures in which PI fluorescence was detected at this stage were excluded from further study. Hypoxia was induced by transferring cultures to SFM (+PI) which had been saturated with 95%N_2/5%CO_2. Culture plates (without lids) were then sealed into an airtight chamber in which the atmosphere was saturated with 95%N_2/5%CO_2 by continuously blowing through gas at 10L/min for ten minutes before being sealed and placed in the incubator for 170mins (total time of hypoxia was therefore 180 mins). At the end of the hypoxic period cultures were returned to normoxic SFM containing PI and placed back in the incubator for 24 hours.

Neuronal damage was assessed as described previously (Pringle *et al.*, 1996; 1997) using either NIH Image 1.60 running on an Apple IIsi computer or OpenLab 2.1 (Improvision) running on a Macintosh G4/400. Images were captured using a monochrome camera and saved onto optical disk for offline analysis. Light transmission images were captured prior to the addition of drugs, and PI fluorescence images recorded at the end of the 24-hour post-hypoxia recovery period. The area of the CA1 cell layer was determined from the transmission image. The area of PI fluorescence in CA1 was measured using the density slice function within NIH Image or OpenLab, and neuronal damage expressed as the percentage of the CA1 in which PI fluorescence was detected above background.

Steroid compounds were prepared by making an initial 1mg/ml solution in ethanol and further diluting down in SFM. Compounds were added to the cultures for 45 minutes prior to hypoxia, during the hypoxic episode and during the post-hypoxic recovery period. Control experiments consisted of cultures treated with vehicle alone.

RESULTS

Experiment 1:

An initial experiment was performed to determine whether 7αOH-EPIA and 7βOH-EPIA were neuroprotective at a high concentration of 100nM. Hypoxia produced a lesion in 25.5±6.4% of CA1. This damage was significantly reduced by both 7αOH-EPIA and 7βOH-EPIA when present pre-, during and post-hypoxia (see table I).

Table 1

Compound	N	% Damage in CA1
Control Hypoxia	17	25.5±6.4
Hypoxia + 100nM 7αOH-EPIA	16	4.0±2.9**
Hypoxia + 100nM 7βOH-EPIA	16	9.0±4.7*

Experiment 2:

Having determined that both the α- and β-isomers of 7OH-EPIA were neuroprotective, we assessed the concentration-dependency of this effect. Control hypoxia resulted in neuronal damage to 31.9±4.7% of the CA1. 7βOH-EPIA was significantly neuroprotective at 10nM and 100nM, but activity was lost if the concentration was reduced to 1nM. as shown in Table II, below.

Table II

Compound	N	% Damage in CA1
Control Hypoxia	29	31.9±4.7
Hypoxia + 1nM 7βOH-EPIA	15	20.6±7.2
Hypoxia + 10nM 7βOH-EPIA	12	11.9±4.7*
Hypoxia + 100nM 7βOH-EPIA	13	14.3±5.0*

Experiment 3:

Having observed the neuroprotective activity of 7βOH-EPIA, we next investigated whether 7βOH-DHEA was neuroprotective. Cultures were incubated with either 100nM 7βOH-DHEA or vehicle, pre-, during and post-hypoxia. Hypoxia produced damage in 29.0±6.2% of CA1. In cultures treated with 7βOH-DHEA, a large, highly significant, reduction in neuronal damage was observed as shown in Table III, below.

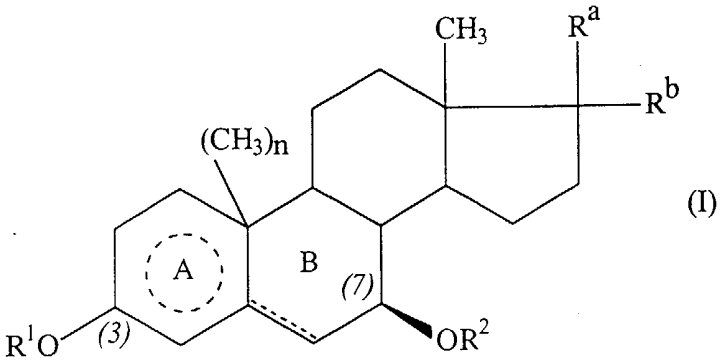
Table III

Compound	N	% Damage in CA1
Control Hypoxia	21	29.0±6.2
Hypoxia + 100nM 7βOH-DHEA	16	4.2±1.9**

CLAIMS

1. The use for the manufacture of a medicament for protection against neuronal damage of a 3-hydroxy-7β-hydroxy steroid or a 3-oxo-7β-hydroxy steroid and pharmaceutically acceptable esters thereof.

2. The use according to Claim 1, in which the steroid is a compound of formula (I):



(I)

wherein

R^1 and R^2 are the same as or different from each other and each represents a hydrogen atom, an alkyl group having from 1 to 6 carbon atoms, an alkenyl group having from 2 to 6 carbon atoms, an alkynyl group having from 2 to 6 carbon atoms, an aryl group having from 6 to 10 carbon atoms, a formyl group, an alkylcarbonyl group having from 2 to 7 carbon atoms, an alkenylcarbonyl group having from 3 to 7 carbon atoms, an alkynylcarbonyl group having from 3 to 7 carbon atoms, an arylcarbonyl group having from 7 to 11 carbon atoms, an aralkylcarbonyl group having from 8 to 15 carbon atoms, an aralkenylcarbonyl group having from 9 to 15 carbon atoms, or a heterocyclic-carbonyl group, as defined below;

one of R^a and R^b represents a group of formula $-R^c$, preferably in the β configuration, and the other represents a hydrogen atom, or R^a and R^b together represent an oxo group;

R^c represents an alkanoyl group having from 1 to 6 carbon atoms, an aryl-carbonyl group, in which the aryl part is an aromatic carbocyclic group having from 6 to 10

ring carbon atoms, a heterocyclic-carbonyl group, as defined below, or a group of formula $-OR^4$, where R^4 represents any one of the groups and atoms defined above for R^1 and R^2;

the ring A, 

, is a benzene or cyclohexane ring;

when ring A is a cyclohexane ring, the dotted line in ring B represents a single or double carbon-carbon bond and n is 1; or when ring A is a benzene ring, the dotted line in ring B represents a single carbon-carbon bond and n is 0;

said heterocyclic-carbonyl group is a group of formula R^3–CO , where R^3 represents a heterocyclic group having from 3 to 7 ring atoms, of which from 1 to 3 are hetero-atoms selected from nitrogen atoms, oxygen atoms and sulphur atoms, and the remaining atom or atoms of which there is at least one is or are carbon atoms;

said alkyl, alkenyl and alkynyl groups and the alkyl, alkenyl and alkynyl parts of said alkylcarbonyl, alkenylcarbonyl and alkynylcarbonyl groups being unsubstituted or having at least one of the following substituents ψ:

substituents ψ: hydroxy groups, mercapto groups, halogen atoms, amino groups, alkylamino groups having from 1 to 6 carbon atoms, dialkylamino groups in which each alkyl group has from 1 to 6 carbon atoms, carbamoyl groups, nitro groups, alkoxy groups having from 1 to 6 carbon atoms, alkylthio groups having from 1 to 6 carbon atoms, carboxy groups, alkoxycarbonyl groups and unsubstituted aryl groups having from 6 to 10 carbon atoms;

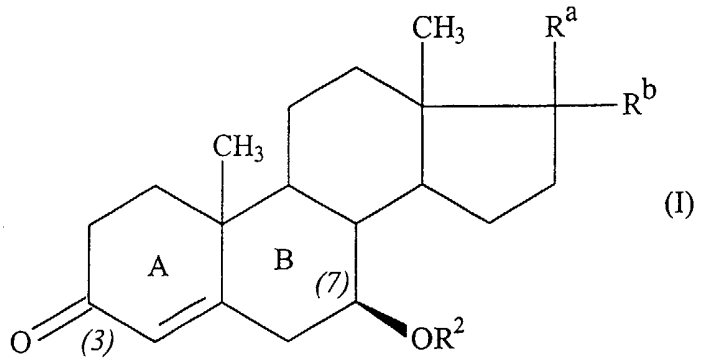
said aryl groups, said heterocyclic groups, and the aryl parts of said arylcarbonyl groups and said aralkylcarbonyl groups being unsubstituted or having at least one of the following substituents β:

substituents ξ: any of substituents ψ, and alkyl groups having from 1 to 6 carbon atoms, hydroxyalkyl groups having from 1 to 6 carbon atoms, and haloalkyl groups

having from 1 to 6 carbon atoms;

and pharmaceutically acceptable salts and esters thereof.

3. The use according to Claim 2, in which the steroid is a compound of formula (II):



(I)

in which R^a, R^b and R^2 are as defined in Claim 2.

4. The use according to Claim 1, in which the steroid is 7β-hydroxy-epiandrosterone.

5. The use according to Claim 1, in which the steroid is 7β-hydroxy-dehydro-epiandrosterone.

6. The use according to Claim 1, in which the steroid is 7β-hydroxy-17β-oestradiol.

7. The use according to Claim 1, in which the steroid is 7β-hydroxy-pregnenolone.

8. The use according to Claim 1, in which the steroid is7β-hydroxy-oestrone.

9. The use according to Claim 1, in which the steroid is7α-hydroxy-oestrone.

INVESTOR IN PEOPLE

Application No: GB 0016027.5
Claims searched: 1-9

Examiner: Annabel Ovens
Date of search: 3 January 2001

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.S): A5B

Int Cl (Ed.7): A61K (31/565, 31/5685, 31/566, 31/57)

Other: Online: PAJ, EPODOC, WPI, TXTE, CAS-ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
Y	GB 2317826 A	(UNIVERSITY OF EDINBURGH) see page 4 lines 15-17	1, 2, 4, 5 and 7
X	WO 97/37664 A2	(BRITISH TECHNOLOGY GROUP LTD) see page 4 line 23-page 5 line 27 and page 11 line 4-page 12 line 25	1, 2 and 5-9
X	WO 94/20111 A1	(UNIVERSITY OF UTAH) see page 13 lines 11-18 and page 16 line 20-page 17 line 22	1, 2 and 5
X	WO 94/03176 A1	(HUMANETICS CORP) see page 2 lines 25-35 and page 3 lines 19-29	1, 2 and 5
Y	US 5763433	(MORFIN) see whole document	1, 2, 4, 5 and 7
Y	Immunol. Cell Biol. Vol. 78, No. 3, 2000, V Chmiewlewski et al., "Dexamethasone-induced apoptosis of mouse thymocytes: prevention by native 7α-hydroxysteroids", pages 238-246		1, 2, 4, 5 and 7

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.