ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 299-313

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

The S-oxidative degradation of a novel corticosteroid tipredane (INN) Part III[1]. Detailed investigations into the disulphoxidation of tipredane

Melvin R. Euerby[a,*], John A. Graham[a,b], Christopher M. Johnson[a], Richard J. Lewis[a], Don B. Wallace[b]

[a]*Pharmacuetical & Analytical Research & Development, Astra Charnwood, Bakewell Road, Loughborough, Leicestershire, LE11 0RH, UK*

[b]*Department of Pharmaceutical Sciences, University of Strathclyde, Royal College, 204 George Street, Glasgow, G11 XW, UK*

Received for review 6 February 1996; revised manuscript recieved 24 April 1996

Abstract

The methyl- and ethylsulphoxide diastereoisomers (**V** and **VI**) of the corticosteroid tipredane (INN, **I**) have been shown to undergo further stereoselective S-oxidation to yield diastereoisomeric disulphoxides (**II**).

Interactive computer optimisation software was employed to develop semi-preparative chromatography conditions for the isolation of the disulphoxide diastereoisomers (**II**) and to develop a multiselective gradient HPLC analysis of tipredane (**I**), the four monosulphoxide diastereoisomeric pairs (**V**, **VI**, **IX** and **X**), the four disulphoxide diastereoisomers (**II**), the vinyl methyl and ethyl derivatives (**XI** and **XII**) and the methylsulphone of tipredane (**VII**). The four diastereoisomeric disulphoxides (**II**) have been isolated by semi-preparative HPLC and their structures unambiguously confirmed by high resolution multinuclear NMR and mass spectrometry. The stereochemical assignment of the four disulphoxide diastereoisomers (**II**), the ethylsulphoxide diastereoisomeric pair (**VI**), and the vinyl methyl and ethylsulphoxide diastereoisomeric paris (**IX** and **X**) was determined by degradation/synthesis and relation to the *S*/*R*-disulphoxide (**II**) whose stereochemistry was determined by X-ray crystallography. The monosulphoxides (**V** and **VI**) showed a high degree of site and stereoselectivity towards further S-oxidation. S-Oxidation on the C-17 β-substituent of tipredane occurred at a rate approximately 50-fold faster than that on the α-substituent.

The disulphoxides (**II**) have been shown to be susceptible to thermolysis yielding the vinyl methylsulphoxide diastereoisomers (**IX**) preferentially. The loss of the ethylsulphenic acid from the disulphoxide diastereoisomers (**II**) could be rationalised in terms of the preferred rotamers of the C-17 substituents.

Keywords: Corticosteroid; Disulphoxide diastereoisomers (epimers); Dithioketal; S-oxidation; Site and stereoselectivity; Tipredane

* Corresponding author. Fax: (+44) 01509-645590; e-mail: mel.euerby@charnwood.gb.astra.com.

[1] For Part II, see Ref. [3].

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PII S0731-7085(96)01860-2

1. Introduction

Tipredane (INN, [11β,17α]-17-[ethylthio]-9α-fluoro-11-hydroxy-17-[methylthio]-androsta-1,4-di-ene-3-one, **I**) is a novel corticosteroid [1], which can undergo non-enzymatic [2,3] and enzymatic [4] S-oxidation of the C-17 asymmetric dithioketal moiety in a site- and stereoselective manner, yielding an array of monosulphoxide diastereoisomers. Tipredane is extremely prone to non-enzymatic S-oxidative degradation when formulated in the presence of excipients, such as polyethylene glycols, which contain peroxides [5].

The authors have previously shown that in the presence of limited quantities of oxidant (relative to tipredane), the major products are the methyl- and ethylsulphoxide diastereoisomers (**V** and **VI**) [2,3]. However, in the presence of excess oxidant, LC/MS studies have indicated that disulphoxide diastereoisomers (**II**) are preferentially formed.

In addition to non-enzymatic S-oxidative degradation, tipredane has been reported to under go a facile enzyme-mediated S-oxidative metabolism, when incubated with rat, mouse and human liver homogenates, to yield a similar array of metabolically-inactive sulphoxide diastereoisomers. The most abundant in-vivo human metabolites were the 6β-hydroxy-16,17-vinylmethylsulphoxide diastereoisomers (**III**), formed in a *S*:*R*-sulphoxide configuration of approximately 9:1, and the corresponding sulphone derivative (**IV**).

As a prerequisite to the study of the site- and stereoselectivity of the disulphoxidation of tipredane, it was essential to develop a chromatographic separation of the disulphoxides, monosulphoxides and elimination products. Therefore, this paper will describe the development of an optimised gradient HPLC methodology. In addition, the use of the computer optimisation technique (ICOS) will be compared and contrasted with that of a manual approach.

In order to unambiguously confirm the identification of the disulphoxide diastereoisomers in the over-oxidation of tipredane, this paper will describe the isolation and spectroscopic characterisation of the four diastereoisomeric disulphoxides (**II**) and the stereochemical assignment of the mono- and disulphoxides, based on X-ray crystallography. Their ability to undergo thermal elimination of alkylsulphenic acids will also be discussed.

As an extension to earlier investigations into the site- and stereoselectivity of the non-enzymatic S-oxidative degradation of tipredane (monosulphoxidation), this paper will report observations on the site- and stereoselectivity of the non-enzymatic diastereoisomeric disulphoxide (**II**) formation (disulphoxidation of tipredane), from the corresponding methyl- and ethylsulphoxide diastereoisomers (**V** and **VI**), which have been postulated as being formed by further non-enzymatic and enzymatic S-oxidation of the tipredane monosulphoxides.

Special emphasis was placed on the site- and stereoselectivity of the disulphoxidation as it was previously reported that the degree of site- and stereoselectivity of the S-oxidation of tipredane observed with differing achiral oxidants could be rationalised in terms of the preferred rotamers of the dialkyl groups of the C-17 position and the steric hindrances imposed on the approach of the varying-size oxidants [3]. It was therefore anticipated that these restrictions would be greater in the case of the S-oxidation of the monosulphoxide diastereoisomers of tipredane.

In addition, the stability of the resulting disulphoxides was examined, as the monosulphoxides have been shown to be extremely prone to elimination of alkylsulphenic acid; if this is also the case for all or certain of the disulphoxide diastereoisomers (**II**), then this may explain the stereoselectivities observed during human in-vivo metabolic inactivation.

The structures of the compounds mentioned in this paper are shown in Fig. 1. The stereochemistry of the disulphoxide diastereoisomers (**II**) follows the convention of describing the configuration of methylsulphoxide/ethylsulphoxide moieties.

The S-Oxidative Degradation of Tipredane. Part III.

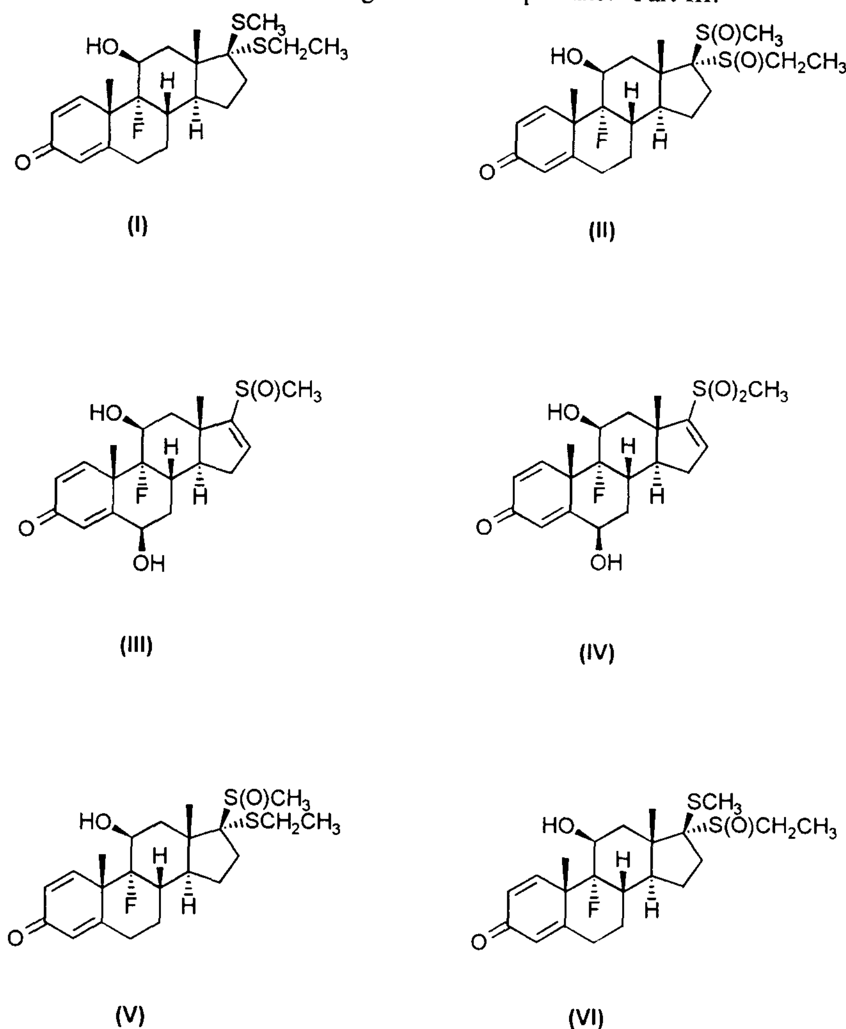

Fig. 1. Structure of tipredane and related compounds. For clarity the stereochemistry of the sulphoxide moieties has not been shown.

2. Experimental

2.1. Chemicals

All chemicals and solvents used were of HPLC grade. Water was purified by an Elgastat spectrum RO and ion exchange/carbon filter system supplied by Elga (High Wycombe, UK). The methyl- and ethylsulphoxide epimers (**V** and **VI** respectively) and the vinyl methyl- and ethylsulphoxide epimers (**IX** and **X** respectively) were prepared according to Euerby and co-workers [2,3]. The methylsulphone (**VII**) was assigned from the chromatographic data previously reported [3].

Melting points (m.p.) were taken on a Linkam THM600 Hot Stage Unit fitted with a Linkam 90 Programmer; samples were analysed in a oxygen-free nitrogen environment, rate of heating: 10°C min^{-1} to 300°C.

2.2. Spectroscopy

NMR spectra were recorded on a Bruker AMX-500 spectrometer operating at 500.138 and 125.759 MHz for proton and carbon nuclei re-

The S-Oxidative Degradation of Tipredane. Part III.

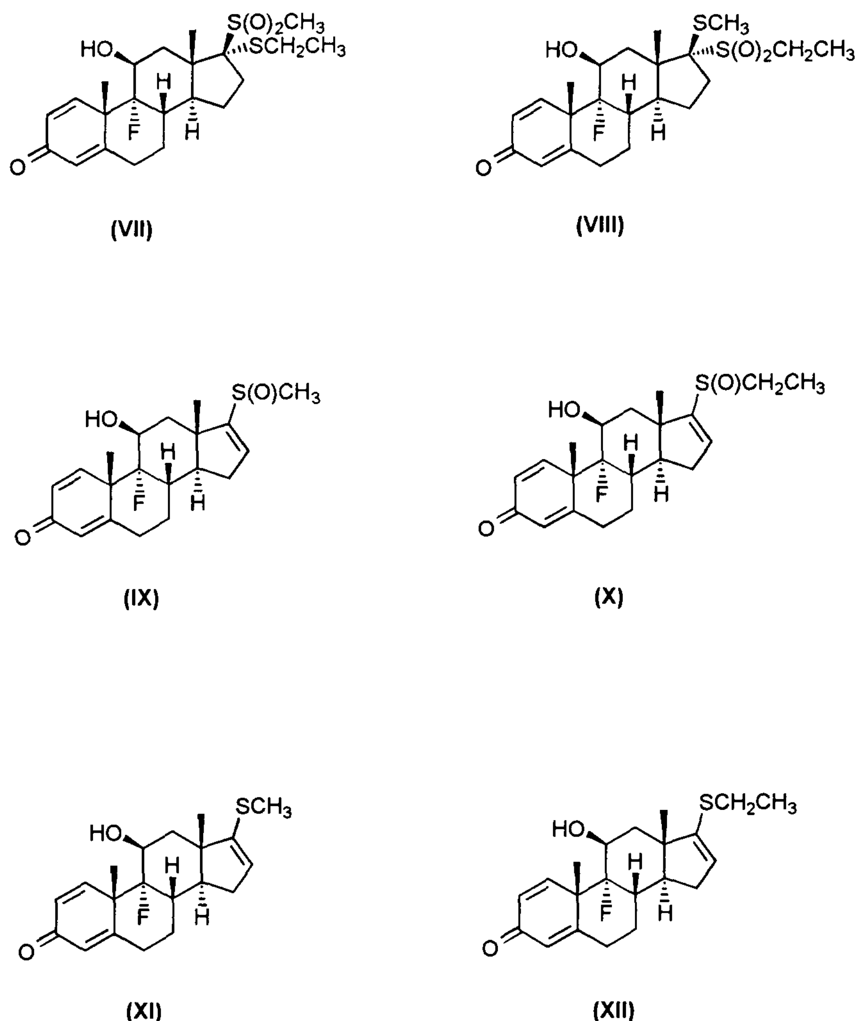

Fig. 1. Contd.

spectively. FT-IR spectra were recorded using KBr discs on a Perkin-Elmer 2000 FT-IR spectrometer. Thermospray (TP-MS) and fast atomic bombardment mass spectroscopic (FAB-MS) techniques were performed on a Finnigan MAT TSQ700, utilising an ammonium acetate (0.4 M)–acetonitrile mobile phase, and a VG 70-250 SEQ mass spectrometer respectively. For FAB-MS the sample was in a matrix of nitrobenzylalcohol and dimethylsulphoxide.

2.3. General chromatographic conditions

HPLC analyses were performed using a Hewlett-Packard 1090M HPLC system equipped with a 1040 linear photodiode arrray UV detector, column oven and ternary solvent delivery system. Data acquisition and integration was controlled by a Hewlett-Packard 79994A Chem Station.

Detection was at 240 nm, based on the λ_{max} value of tipredane. The flow rate was 1.5 ml min^{-1}, unless otherwise stated.

Reaction products were identified by comparison of retention times, by spiking with authentic materials and by UV diode array spectroscopy. Calculations assumed equal relative response factors. This assumption is believed to be valid because the absorbance at 240 nm is due to the A ring of the steroid and is present in all of the compounds investigated.

The HP 79979A ICOS comprised three packages: (1) elution strength selection (for the determination of isoeluotropic mobile phase compositions); (2) lattice search (for acquiring optimisation and selectivity data); and (3) retention modelling (for interpreting the data).

2.4. ICOS optimisation investigations

The ICOS optimisation procedure for the analytical investigation was performed on a 3 μm Hypersil Excel ODS (150 mm × 4.6 mm, Hichrom Ltd., Reading, UK) column. The oven temperature was thermostatically held at 40°C and a flow rate of 1 ml min^{-1} was used. The elution strength selection was performed using binary mixtures of ammonium acetate buffer (0.025 M, pH 7.1) with methanol, acetonitrile and tetrahydrofuran. The desired retention time for the last-eluting peak was set at 14 min.

The ICOS optimisation procedure for the semi-preparative investigations was performed on a 5 μm Ranin Dynamax-60 Å C-18 semi-preparative axial compression column (250 mm × 4.6 mm, Anachem Ltd., Luton, UK) fitted with a Dynamax C-18 guard column (8 μm, 50 mm × 4.6 mm). The oven temperature was maintained at ambient temperature with a flow rate of 0.8 ml min^{-1}. The elution strength selection was performed using binary mixtures of ammonium acetate buffer (0.025 M, pH 7.1) with methanol, acetonitrile and tetrahydrofuran. The desired retention time for the last-eluting peak, for the semi-preparative investigation, was set at 16 min.

2.5. HPLC conditions for the separation of the disulphoxide diastereoisomers (II)

Chromatography was performed on a 3 μm Hypersil Excel ODS (150 mm × 4.6 mm, Hichrom Ltd.) column. The eluent consisted of mobile phases A and B which were ammonium acetate (0.025 M, pH 7.1) in tetrahydrofuran–methanol–acetonitrile–water (4.8:16:4:75.2 v/v) and ammonium acetate (0.025 M, pH 7.1) in acetonitrile–water (65:35 v/v) respectively. The oven temperature was thermostatically held at 40°C. After a 20 min isocratic period of 0% mobile phase B, a linear gradient was run over 20 min to 100% mobile phase B; then the eluent composition was held for a further 10 min.

2.6. Semi-preparative HPLC

This was performed using three Gilson 305 pumps connected to a Gilson 811B Dymanic Mixer and Gilson 806 Manometric Module (Anachem Ltd.). The pumps were controlled by a Gilson gradient HPLC system. The column effluent was continuously monitored at 270 nm using a Gilson 115UV detector. Data acquisition and integration were controlled by an IBM computer using Gilson Microwindow software. Fractions were collected manually using the detector as a monitor. The third pump was used to introduce the sample solution onto the column. Chromatography was performed on a 5 μm Ranin Dynamax-60 Å C-18 semi-preparative axial compression column (250 mm × 21.4 mm, Anachem Ltd.) fitted with a Dynamax C-18 guard column (8 μm, 100 mm × 21.4 mm). The eluent consisted of tetrahydrofuran–methanol–acetonitrile–water (2:32.5:6.5:59 v/v) at a flow rate of 17 ml min^{-1}.

2.7. Reaction of tipredane (I) with monomagnesium peroxyphthalic acid (MMPP) and isolation of the diastereoisomeric disulphoxides (II)

MMPP (3.016 g, 4.878 mmoles) in ammonium acetate buffer (0.5 M, pH 7.1, 500 ml) was added dropwise to a solution of tipredane (**I**, 2.002 g, 4.876 mmoles) in ethanol (500 ml) at 4°C. The reaction was monitored using the disulphoxide HPLC conditions. Prior to analysis each aliquot was diluted 50:50 with ammonium acetate buffer (0.5 M, pH 7.1). After completion of the reaction (approximately 30 min), ice-cold water (800 ml)

was added to portions of the reaction mixture (200 ml) to bring the ethanolic content down to 10% v/v. The 10% v/v ethanolic fraction (1000 ml) was then passed through a Mega-Bond Elut C-18 column (60 ml, Analytichem Ltd.), which had been previously conditioned with ethanol (25 ml), water (25 ml) and 10% v/v ethanol–water (50 ml). After loading, the column was eluted with 10% v/v ethanol–water (100 ml) to remove phthalic acid. The disulphoxides were then eluted from the column with 20% v/v ethanol (2 × 500 ml). This procedure was repeated for the remaining diluted reaction mixture (4 × 1000 ml). The combined 20% v/v ethanolic solution (5 × 1000 ml) of the disulphoxides was evaporated under reduced pressure (Büchi RE111 Rotavapor plus Büchi 461 Water Bath) at < 40°C to one-fifth of its volume (1000 ml). The concentrated disulphoxide solution was separated using semi-preparative HPLC by loading 15 × 50 ml of the above solution via the third pump of the Gilson semi-preparative HPLC system. Heart-cut fractions of the second (*S*/*R*), third (*S*/*S*) and fourth (*R*/*R*) peaks were collected in order to achieve the desired purity. It was not possible to isolate the third and fourth peaks to the required purity (> 95%) at the first pass; therefore, the organic solvents from the fraction were evaporated under reduced pressure at < 40°C and subjected to further semi-preparative HPLC.

The organic solvent from the pure disulphoxide fractions was evaporated under reduced pressure at < 40°C (volume reduced by 40%) and then loaded onto a pre-conditioned Mega-Bond Elut column [60 ml, Analytichem; conditioned with ethanol (50 ml), water (50 ml) and 5% v/v ethanol–water (50 ml)], washed with water (50 ml) and the pure disulphoxide eluted with ethanol (100 ml) and evaporated under reduced pressure at < 40°C to yield the disulphoxides as white crystals. Non-optimised yields are quoted. The disulphoxides were found to exhibit complex thermal events prior to decomposition at temperatures in the region of 275°C.

*2.7.1. [11β,17α]-17[S-ethylsulphinyl]-9α-fluoro-11β-hydroxy-17[R-methylsulphinyl]androsta-1,4-diene-3-one (R/S-**II**)*

Yield: 120 mg (7.5%); ν_{max} (KBr; cm^{-1}): 3400(OH), 1660(1,4-dien-3-one), 1060 (C–F), 1056 and 1011 (S–O); δ_H (d_6-DMSO): 7.28 (1H,d,J = 10, 1-H), 6.23 (1H,dd,J = 10 and 2,2-H), 6.02 (1H,d,J = 2,4-H), 5.47 (1H,s,11-βOH), 4.19 (1H,m, 1, 11-H), 3.27 and 3.06 (2 × 1H, 2 × m,21-CH_2), 3.02 (3H,s,20-Me), 2.64 and 2.35 (2 × 1H, 2 × m, 6α/β-H), 2.50 (1H,m,8-H), 2.47 and 2.16 (2 × 1H, 2 × m, 12α/β-H), 2.38 and 1.85 (2 × 1H, 2 × m, 16α/β-H), 2.08 (1H,m,14-H), 1.83 and 1.37 (2 × 1H, 2 × m, 7α/β-H), 1.73 and 1.50 (2 × 1H, 2 × m, 15α/β-H), 1.51 (3H,s,19-Me), 1.48 (3H,s,18-Me), 1.23 (3H,t,J = 17, 22-Me); δ_c (d_6-DMSO): 185.0 (s,C-3), 166.3 (s,C-5), 152.3 (s,C-1), 128.8 (s,C-2), 124.0 (s,C-4), 100.4 (d,J_{CF} = 175,C-9), 83.4 (s,C-17), 70.2 (d,J_{CF} = 36,C-11), 51.1 (s,C-13), 47.5 (d,J_{CF} = 23,C-10), 46.7 (s,C-14), 41.7 (s,C-21), 38.5 (s,C-12), 37.0 (s,C-20), 33.1 (d,J_{CF} = 19,C-8), 29.9 (s,C-6), 27.0 (s,C-7), 25.8 (s,C-16), 23.5 (s,C-15), 22.6 (d,J_{CF} = 5,C-19), 17.0 (s,C-18), 8.4 (s,C-22); MS m/z (FAB, %): 443 (M^+ + 1, 11), 365 (M^+ – EtSOH, 2), 301 (M^+ – EtSOH and MeSOH, 9), 289 (14), 154 (100), 136 (72); HPLC: R_t = 19 min and purity = 99.2%.

*2.7.2. [11β,17α]-17[R-ethylsulphinyl]-9α-fluoro-11β-hydroxy-17[S-methylsulphinyl]-androsta-1,4-diene-3-one (S/R-**II**)*

Yield: 134.5 mg (8.3%); ν_{max} (KBr; cm^{-1}): 3400 (OH), 1660 (1,4-dien-3-one), 1060(C–F), 1070 and 1016(S–O); δ_H (d_6-DMSO):7.28 (1H,d,J = 10,1-H), 6.22 (1H,dd,J = 10 and 2,2-H), 6.01 (1H,d,J = 2,4-H), 5.47 (1H,s,11-βOH), 4.18 (1H,m,11-H), 3.32 and 3.28 (2 × 1H, 2 × m, 21-CH_2), 2.78 (3H,s,20-Me), 2.61 and 2.28 (2 × 1H, 2 × m, 6α/β-H), 2.50 (1H,m,8-H), 2.31 and 1.95 (2 × 1H, 2 × m, 12α/β-H), 2.28 and 2.25 (2 × 1H, 2 × m, 16α/β-H), 2.20 (1H,m, 14-H), 1.82 and 1.62 (2 × 1H, 2 × m, 7α/β-H), 1.77 and 1.53 (2 × 1H, 2 × m, 15α/β-H), 1.49 (3H,s,19-Me), 1.24 (3H,s,18-Me), 1.23 (3H,t,J = 7,22-Me); δ_C (d_6-DMSO):185.0 (s,C-3), 166.0 (s,C-5), 152.3 (s,C-1), 128.9 (s,C-2), 124.0(s,C-4), 100.4(d,J_{CF} = 175,C-9), 82.2(s,C-17), 70.4(d,J_{CF} = 36,C-11), 48.5(s,C-13), 47.5 (d,J_{CF} = 23,C-10), 45.7 (s,C-14), 42.2 (s,C-21), 36.3 (s,C-12), 34.7 (s,C-20), 33.1 (d,J_{CF} = 19,C-8), 30.0 (s,C-6), 27.1 (s,C-7), 24.3

(s,C-15), 22.5 (d,J_{CF} = 5,C-19), 22.2 (s,C-16), 18.7 (s,C-18), 8.0 (s,C-22); MS m/z (FAB, %): 443 (M^+ + 1, 15), 365 (M^+-EtSOH 10), 301(M^+ − EtSOH and MeSOH, 25), 289 (15), 154 (100), 136 (75); HPLC:R_t = 21.5 min and purity = 98.2%.

*2.7.3. [11β,17α]-17[S-ethylsulphinyl]-9α-fluoro-11β-hydroxy-17[S-methylsulphinyl]androsta-1,4-dene-3-one (S/S-**II**)*

Yield: 71.5 mg (4.4%); ν_{max} (KBr; cm^{-1}): 3400 (OH), 1660 (1,4-dien-3-one), 1060 (C–F), 1061 and 1037 (S–O);δ_H (d_6-DMSO): 7.30 (1H,d,J = 10,1-H), 6.24 (1H,dd,J = 10 and 2,2-H), 6.02 (1H,d,J = 2,4-H), 5.44 (1H,s,11-βOH), 4.23 (1H,m,11-H), 4.15 and 3.24 (2 × 1H, 2 × m, 21-CH_2), 3.31 (3H,s,20-Me), 2.65 and 2.35 (2 × 1H, 2 × m, 6α/β-H), 2.63 and 2.07 (2 × 1H, 2 × m, 12α/β-H), 2.54 (1H,m,8-H), 2.49 (2H,m,16α/β-H), 1.95 (1H,m,14-H), 1.83 and 1.40 (2 × 1H, 2 × m, 7α/β-H), 1.71 and 1.50 (2 × 1H, 2 × m, 15α/β-H), 1.51 (3H,s,19-Me), 1.24 (3H,s,18-Me), 1.19 (3H,t,J = 7, 22-Me); δ_C (d_6-DMSO):185.0 (s,C-3), 166.2 (s,C-5), 152.2 (s,C-1), 128.9 (s,C-2), 124.2 (s,C-4), 100.4 (d,J_{CF} = 175,C-9), 80.2 (s,C-17), 70.2 (d,J_{CF} = 36,C-11), 48.4 (s,C-13), 47.5 (d,J_{CF} = 23,C-10), 46.0 (s,C-14), 39.7(s,C-21), 37.8(s,C-12), 34.7(s,C-20), 33.0(d,J_{CF} = 19,C-8), 29.9(s,C-6), 27.9(s,C-6), 27.1 (s,C-7), 23.9 (s,C-15), 22.6 (d,J_{CF} = 5,C-19), 17.5(s,C-18), 9.6(s,C-22); MS m/z (FAB, %):443 (M^+ + 1, 19), 365 (M^+ − EtSOH, 6), 301 (M^+ − EtSOH and MeSOH, 12), 289 (15), 154 (100), 136 (71); HPLC:R_t = 24 min and purity = 85% (first purification) and 98.6% (second purification).

*2.7.4.[11β,17α]-17[R-ethylsulphinyl]-9α-fluoro-11β-hydroxy-17[R-methylsulphinyl]androsta-1,4-diene-3-one (R/R-**II**)*

Yield: 60.8 mg (3.8%); ν_{max} (KBr; cm^{-1}): 3400 (OH), 1660 (1,4-dien-3-one), 1060 (C–F), 1072 and 1008 (S–O); δ_H (d_6-DMSO): 7.25 (1H,d,J = 10,1-H), 6.21 (1H,dd,J = 10 and 2,2-H), 6.02 (1H,d,J = 2,4-H), 5.63 (1H,s,11-βOH), 4.14 (1H,m,11-H), 3.85 and 3.69 (2 × 1H, 2 × m, 21-CH_2), 2.95 (3H,s,20-Me), 2.64 and 2.33 (2 × 1H, 2 × m, 6α/β-H), 2.52 (1H,m,8-H), 2.45 and 1.63 (2 × 1H, 2 × m, 16α/β-H), 2.00 (1H,m,14-H), 1.91 and 1.69 (2 × 1H, 2 × m, 12α/β-H), 1.86 and 1.38 (2 × 1H, 2 × m, 7α/β-H), 1.83 (2H,m,15α/β-H), 1.53 (3H,s,19-Me), 1.50 (3H,s,18-Me), 1.33 (3H,t,J 7, 22-Me); δ_C (d_6-DMSO): 185.0 (s,C-3), 166.2 (s,C-5), 152.1 (s,C-1), 128.9 (s,C-2), 124.1 (s,C-4), 100.6 (d,J_{CF} = 175,C-9), 82.0 (s,C-21), 69.8(d,J_{CF} = 36,C-11), 48.4(s,C-13), 47.5(d,J_{CF} = 23,C-10), 46.2(s,C-14), 42.2(s,C-21), 37.4 (s,C-12), 32.4 (s,C-20), 33.6 (d,J_{CF} = 19,C-8), 29.9 (s,C-6), 27.2 (s,C-7), 24.0 (s,C-15), 23.6 (s,C-16), 22.5 (d,J_{CF} = 5,C-19), 18.0 (s,C-18), 10.1 (s,C-22); MS m/z (FAB, %): 443 (M^+ + 1,7), 301 (M^+ − EtSOH and MeSOH, 8), 289 (12), 154 (100), 136 (67); HPLC:R_t = 27.5 min and purity = 89% (first purification and 98.4% (second purification).

2.8. Preparation of crystals suitable for X-ray crystallography

Crystals were grown by the vapour diffusion methodology as described previously [6]. Each of the four disulphoxide diastereoisomers (**II**, ≈ 2 mg) were dissolved in ethyl acetate (< 1 ml) and filtered. These solutions, in open glass vials, were placed in a closed glass bottle containing a small volume (≈ 2 ml) of petroleum ether (40:60). After 4 days at 4°C, crystals were observed for the S/R and S/S disulphoxide diastereoisomers (**II**). These were filtered and washed with ice-cold ethyl acetate–petroleum ether (40:60) 1:1 v/v. Microscopic examination of the crystals showed that those of the S/R diastereoisomer were of a suitable quality for X-ray crystallographic examination.

3. Results and discussion

Tipredane (**I**) has previously been shown to undergo non-enzymatic [2,3,5] and enzymatic [4] monosulphoxidation in a site- and stereoselective manner, the degree of each being dictated by the steric bulk of the attacking oxidant and the preferred conformations of the tipredane rotamers around the C-17 position [3]. Therefore, it was postulated that the S-oxidation of the methyl- and the ethylsulphoxide diastereoisomers (**V** and **VI**

respectively), which was known to occur in the presence of excess oxidant, may show a greater degree of site- and stereoselectivity due to the increased steric bulk around the C-17 position. Reaction monitoring using a non-optimal HPLC method [2] indicated that the individual *S* and *R*-methylsulphoxide diastereoisomers of tipredane, prepared by the method of Euerby et al. [2], on reaction with MMPP [7] yielded the *S*- and *R*-methylsulphoxide *S*/*R* ethylsulphoxide diastereoisomeric pairs in a stereoselective manner (see Figs. 2a and 2b). The HPLC conditions described in Part I [2] separated the individual diastereoisomeric pairs; however, the method failed to separate all four of the diastereoisomeric disulphoxides (**II**).

*3.1. Separation of the disulphoxide diastereoisomers (**II**)*

In order to separate a mixture of the disulphoxide diastereoisomeric pairs generated from the *S*- and *R*-methylsulphoxides (this mixture gave differing peak area ratios for all the peaks, thus facilitating easier peak tracking), or from tipredane itself, a manual optimisation approach was used using the statistical design for selecting an optimum solvent mixture as described by Glajch et al. [8]. The statistical design took the form of a two-dimensional lattice search comprising of seven experiments using a Hypersil Excel ODS, 150 mm × 4.6 mm, 3 μm, column to exploit the differences in selectivity between acetonitrile, methanol and tetrahydrofuran mixtures. Ammonium acetate 0.025 M pH 7.1 was used, as it was previously observed that the monosulphoxides (**V** and **VI**) were degraded in acidic conditions [3]. Baseline separation of the four diastereoisomeric sulphoxides could be achieved with a run time of 14 min (peaks 1 and 4 = *R*-MeS(O) disulphoxides and peaks 2 and 3 = *S*-MeS(O) disulphoxides) using a quaternary solvent system comprised of ammonium acetate (0.025 M, pH 7.1) in tetrahydrofuran–methanol–acetonitrile–water (5:16:4:75 v/v). Subsequently, these results were compared with those obtained using the HP ICOS approach and they were found to be in excellent agreement, i.e. with ammonium acetate (0.025 M, pH 7.1) in tetrahydrofuran–methanol–acetonitrile–water (5.5:16:2:76.5 v/v).

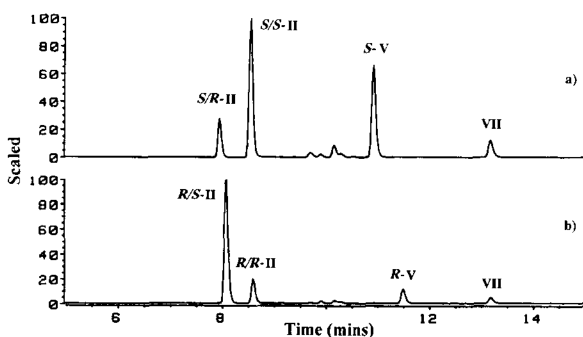

Fig. 2. Reaction of *S*-methylsulphoxide of tipredane (*S*-V) with potassium peroxymonosulphate (30 min at 4°C), monitoring with non-optimised HPLC conditions as described in Ref. [2]. Peaks eluting between 9.5 and 10.5 min have been shown by LC/MS to correspond to the mixed sulphone/sulphoxide diastereoisomers. (b) As (a) except reaction of *R*-methylsulphoxide of tipredane (*R*-V).

*3.2. Multiselective gradient elution HPLC metholdology designed to separate the disulphoxides (**II**), monosulphoxides (**V** and **VI**), sulphones (**VII** and **VIII**) and elimination products (**XI**, **XII** and **IX**, **X**)*

In order to investigate the elimination of alkylsulphenic acids from the various sulphoxides it was essential to develop an HPLC method capable of separating the above compounds. Due to their widely differing polarities, a multiselective gradient elution programme was required. This consisted of an initial isocratic phase corresponding to the optimised quaternary mobile phase composition required to separate the polar disulphoxide diastereoisomers (**II**), followed by an acetonitrile gradient to change the solvent strength and selectivity of the mobile phase in order to achieve good separation and elution of the moderately polar monosulphoxide diastereoisomers (primary S-oxidation products **V** and **VI**), sulphones (secondary S-oxidation products **VII** and **VIII**) and vinylalkylsulphoxide diastereoisomers (**IX** and **X**, elimation products of disulphoxides). Finally a high acetonitrile–aqueous

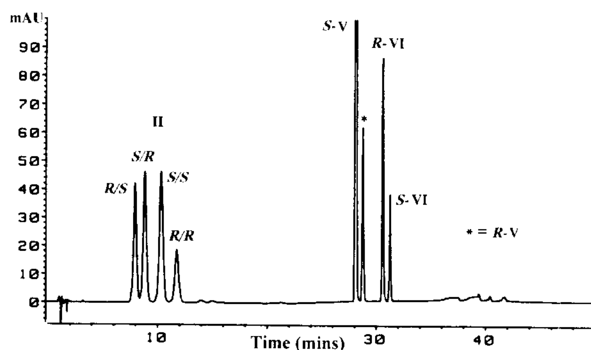

Fig. 3. Resolution of the diastereoisomeric disulphoxides (**II**), methylsulphoxides (**V**) and ethylsulphoxides (**VI**) using the manual optimised multiselective gradient HPLC conditions (see Section 2). The following compounds are additionally separated using this methodology: *S*- and *R*-**X**, $R_t = 23.7$ and 26.3 min respectively; **XI**, $R_t = 39$ min; **XII**, $R_t = 41$ min; **VII**, $R_t = 32.1$ min; **I**, $R_t = 42$ min; *S*- and *R*-**IX**, $R_t = 14$ and 15 min respectively.

composition was maintained to elute the non-polar vinylalkylthioethers (**XI** and **XII**, elimination products of the monosulphoxides), see Fig. 3.

3.3. Semi-preparative mobile phase optimisation

In order to unambiguously confirm the production of each of the disulphoxide diastereoisomers (**II**), a semi-preparative HPLC method was required to enable sufficient quantities to be isolated for spectroscopic analysis. This was develped using the Hewlett Packard (ICOS) computer optimisation software. The three separate programs associated with this software are described below.

3.3.1. Iterative elution strength selection of binary mixtures

The required analysis time was set at 16 min and a flow rate of 0.8 ml min^{-1} was employed (reduced flow rate as high pressures were experienced with the binary methanol mobile phase composition). The iterative elution strength search/program, which was based on the linear relationship of ln(k) to the organic content in the mobile phase, gave the following experimentally derived isoeluotropic mobile phases: acetonitrile–buffer 26:74; methanol–buffer 50:50 (v/v); and tetrahydrofuran–buffer 18:82 (v/v). These compositions were used as the apexes for the lattice solvent selectivity triangle, eluting the last disulphoxide diastereoisomer within 16 min ± 5%. As expected, the three isoeluotropic solvent compositions derived with the 5 μm Ranin Dynamax C-18 column (250 mm × 4.6 mm i.d.) were found to be different to the analytical results which utilised a 3 μm Hypersil Excel ODS column (150 mm × 4.6 mm i.d.). The acetonitrile and tetrahydrofuran binary composition partially separated the four disulphoxide diastereoisomers (**II**), whereas with the methanol binary composition, co-elution of the *S/R* and *S/S* disulphoxide diastereoisomers was observed. In contrast, the analytical optimisation gave reasonable separation of the four disulphoxide diastereoisomers with tetrahydrofuran and methanol binary mixtures. A possible reason for these differences was that a different isoeluotropic plane and hence a different elution strength, with differing selectivity, had been used to elute the last component within the desired analysis time.

3.3.2. Lattice search

In order to describe the resolution surface a total of 15 experiments (three binary, nine ternary and three quaternary mobile phase compositions) were performed for the lattice search evaluation of the data by the retention modelling programme. The disulphoxide diastereoisomers exhibited extremely long retention times when ternary and quaternary mobile phase compositions were examined. For example, a ternary composition comprising a 50:50 mixture of the acetonitrile–methanol binary compositions eluted the last peak at 34 min, compared to 16 min for both the binary mixtures (see Fig. 4). Hence, ln(k) was no longer linearly related to the organic content of the mobile phase. This effect has been observed with the monosulphoxide diastereoisomers of tipredane (**V** and **VI**) [9]. In contrast, the non-polar analyte amylbenzene did not show this trend. The non-linear behaviour of the sulphoxide could possibly be explained in terms of solvent–solvent interactions and solvation differences with the analyte and the stationary phase with different mobile phase compositions.

3.3.3. Retention modelling

Identification of the analyte retention time (peak tracking) was performed by peak area ratios as there was no difference in the ultraviolet spectra of the individual disulphoxide diastereoisomers (**II**). The quality of the separation was based on $R_{s_{min}}$, which was defined as the resolution of the least-separated pair of diastereoisomers. The surface of the triangle, i.e. resolution surface, was examined slice-by-slice through the triangle (total of 10 slices); a disadvantage of the software was that a three dimensional or contour plot of the resolution surface could not be presented using the current software.

A crossover in selectivity was observed in the tetrahydrofuran–acetonitrile ternary mixture slice, i.e. peaks 1 and 2 (*R*/*S* and *S*/*R* diastereoisomer) exhibited peak crossover as more acetonitrile was incorporated into the mobile phase (see Fig. 5).

The model predicted that the optimum separation would be achieveable with a quaternary mixture consisting of acetonitrile–methanol–tetrahydrofuran binary mixtures in a ratio of 25:63:12. Two further experimental determinations were performed near this optimum and the retenion model was updated until the simulated chromatogram mirrored the experimentally derived one. The optimum mobile phase composition corresponded to a 25:65:10 mixture of the binary isoelutropic compositions (equating to acetonitrile–methanol–tetrahydrofuran–buffer 6.5:32.5:1.8:59.2 v/v). An $R_{s_{min}}$ of 2.0 between peaks 3 and 4 with the last peak eluting at approximately 24 min was obtained under these conditions (see Fig. 6). This was in contrast with the optimised analytical method where peaks 1 and 2 exhibited the minimum resolution; however, the same elution order was noted.

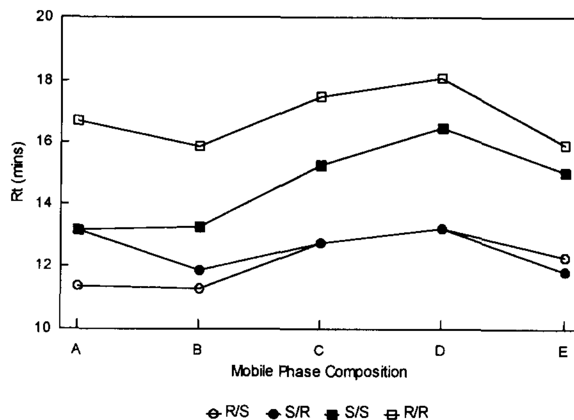

Fig. 5. Change in elution order of the disulphoxide diastereoisomers (**II**) as a function of ternary compositions of acetonitrile–tetrahydrofuran mixtures on a Ranin Dynamax C-18 analytical scout column. Mobile phase composition: A [THF–NH_4OAc buffer (0.025 M, pH 7) (18:82 v/v)]; B [A–E (75:25 v/v)]; C [A–E (50:50 v/v)]; D [A–E (25:75 v/v)]; E [MeCN–NH_4OAc buffer (0.025 M pH 7) (24:76 v/v)] (see Section 2.4).

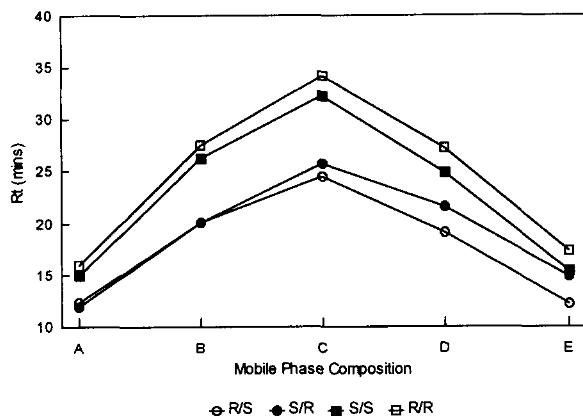

Fig. 4. Effect of the ternary compositions of acetonitrile–methanol mixtures on a Ranin Dynamax C-18 analytical scout column on the retention time of the disulphoxide diastereoisomers (**II**). Mobile phase composition:A [MeCN–NH_4OAc buffer (0.025 M, pH 7) (24:76 v/v)]; B [A–E (75:25 v/v)]; C [A–E (50:50 v/v)]; D [A–E (25:75 v/v)]; E [MeOH–NH_4OAc buffer (0.025 M, pH 7) (50:50 v/v)] (see Section 2.4).

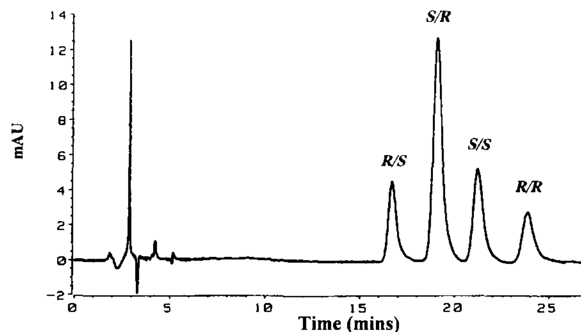

Fig. 6. Resolution of the diastereoisomeric disulphoxides (**II**) using the optimised isocratic HPLC conditions for semi-preparative chromatography (see Section 2).

The possibility of using water in place of a buffer solution for semi-preparative chromatography was investigated. No difference in the chromatography was observed; therefore, for the sake of simplicity, water was used in place of the buffered systems. The only concern was that the disulphoxides may have been unstable in the unbuffered system; this concern was later shown to be unfounded.

*3.4. Synthesis and isolation of the diastereoisomerically pure disulphoxides (**II**)*

In order to generate sufficient quantities of the disulphoxide diastereoisomers (**II**) to be able to unambiguously confirm their structure and to assign their stereochemistry, the most appropriate starting material would be the methylsulphoxide diastereoisomers (**V**) of known stereochemistry. These are extremely liable to thermolysis [2,3] and therefore it was decided to synthesize the disulphoxides (**II**) from tipredane (**I**), which has an enhanced stability profile [2], and to subsequently isolate the disulphoxide diastereoisomers (**II**) by semi-preparative HPLC.

The reaction of tipredane (**I**) with two mole equivalents of MMPP [7] at 4°C in a 1:1 mixture of ethanol and acetate buffer pH 7 was essentially complete within 20 min. The reaction mixture was diluted with water to produce a 10% v/v ethanol–water solution and subsequently passed through a C-18 solid-phase extraction cartridge in order to remove phthalic acid and inorganic ions. Over 90% of the total yield of the disulphoxide diastereoisomeric mixture could be eluted off the cartridge with a purity of greater than 90% (the major contaminant being the vinylalkylsulphoxides [**IX** and **X**]).

The eluent containing the disulphoxide diastereoisomers (**II**) was evaporated. The disulphoxide diastereoisomers were subsequently isolated using the optimised semi-preparative HPLC conditions. The heart-cut technique yielded the *R*/*S*-(peak 1) and *S*/*R*-(peak 2) disulphoxides with purities in excess of 98%. The purity of the *S*/*S* and *R*/*R* fractions (peaks 3 and 4, 85% and 89% respectively) necessitated re-chromatographing these fractions in order to obtain purities in excess of 98%. Due to the potential thermal instability of the disulphoxides (**II**) their solutions were evaporated at a temperature of less than 40°C. Water was removed by adsorbing the compounds onto C-18 solid-phase cartridges and then eluting them with pure ethanol followed by evaporation at a temperature of less than 40°C. The *S*/*R* and *S*/*S* disulphoxides precipitated out of solution during the last evaporation stage whereas the *R*/*S* and *R*/*R* disulphoxides slowly crystallised during storage at −18°C. The pure diastereoisomeric disulphoxides were then unambiguously characterised by high-resolution multinuclear NMR, FT-IR and MS.

*3.5. Spectroscopic characterization of the disulphoxide diastereoisomers (**II**)*

FAB-MS produced an MH^+ ion at m/z 443 for all the disulphoxide (**II**) diastereoisomers. In a similar manner to the methyl- and ethylsulphoxides (**V** and **VI**), the disulphoxide diastereoisomers underwent rapid fragmental loss of methyl- and ethylsulphenic acid in the mass spectrometer.

The ^{1}H and ^{13}C NMR spectroscopic data for the diastereoisomeric disulphoxides (**II**) were essentially as described for the *S*-methylsulphoxide (**V**) [2]. The assignment of the protons and carbon nuclei was based on $^{1}H-^{1}H$ COSY (COrrelated SpectroscopY), HETCOR ($^{1}H-^{13}C$ correlated spectroscopy) and ^{13}C DEPT (Distortionless Enhancement by Polarization Transfer). In order to assign the α and β protons, nOe (nuclear Overhauser) experiments would have been needed. As expected, the chemical shifts of the proton and carbon nuclei in close proximity to the disulphoxide moiety were dependent on the stereochemical configuration of the sulphoxide.

*3.6. Stereochemical characterization of the disulphoxide diastereoisomers (**II**)*

In order to unambiguously establish the stereochemistry of the four disulphoxide diastereoisomers (**II**), the individual monosulphoxide diastereoisomers (**V** and **VI**) were reacted with MMPP and the resultant pair of disulphox-

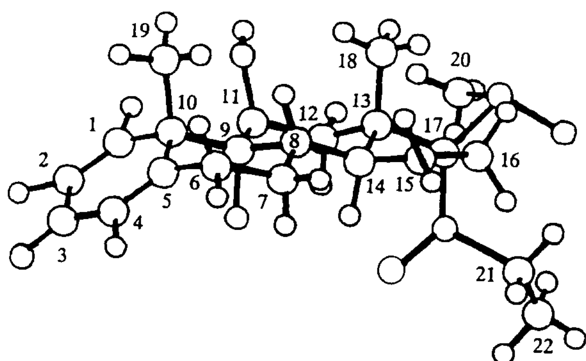

Fig. 7. X-ray crystallographic structure of the S/R-disulphoxide of tipredane (**II**).

ide diastereoisomers (**II**) were analysed on the optimised disulphoxide HPLC screen. The stereochemistry of the monomethylsulphoxide diastereoisomers (**V**) was known [2]; therefore, the stereochemistry of the methylsulphoxide moiety could be assigned in the resultant disulphoxides, assuming that inversion of the optical configuration had not occurred.

The X-ray configuration of the disulphoxide peak 2 was shown to possess the S-methylsulphoxide/R-ethylsulphoxide configurations (see Fig. 7). It has been shown that this disulphoxide is produced from the S-methylsulphoxide (**V**) and the more polar of the ethylsulphoxide diastereoisomers (**VI**); therefore, the R configuration could be assigned to the latter sulphoxide. From the knowledge of which monosulphoxide (**V** or **VI**) generated the corresponding disulphoxide diastereoisomers (**II**) it was possible to establish by a process of inference the configuration of all the disulphoxide (**II**) and monosulphoxide diastereoisomers (**V** and **VI**). The R and S configurations at the sulphoxide moiety could be assigned to the ethylsulphoxide diastereoisomers (**VI**) eluting at 7.5 and 8.4 min respectively using the optimised HPLC method [3]. In addition, the configuration of the vinyl monosulphoxide (**IX** and **X**) could be established from which one was produced during the thermal elimination of ethyl- or methylsulphenic acid from the disulphoxides of known configurations (see later).

The secondary S-oxidation of tipredane (**I**) can, in theory, give rise to four diastereoisomeric disulphoxides (**II**) and the methyl-(**VII**) and ethylsulphones (**VIII**); however, in practise it has not been possible to establish the production of the ethylsulphone. This may be due to the fact that secondary S-oxidation is much faster on the β-substituents of tipredane (**I**) than on the α-face or that the product undergoes 100% elimination of ethylsulphinic acid during chromatographic analysis to yield the vinyl methlythio derivative (**XI**). The latter is unlikely as the corresponding methylsulphone (**VII**) can be successfully chromatographed using similar HPLC conditions [3]. Previously the authors have reported preliminary investigations into the determination of the rate constants for the S-oxidation of tipredane [10] (see Fig. 8 and Table 1 for a summary of these results). The investigations have shown that the secondary S-oxidation of the ethylsulphoxide diastereoisomers (**VI**) appeared to be approximately 50-fold faster than that for the methylsulphoxides (**V**). This was consistent with the results observed for tipredane (**I**) when it was found that oxidation on the β-plane was faster than oxidation on the α-plane.

The R-ethylsulphoxide (**VI**) predominantly formed the S/R disulphoxide diastereoisomer (**II**), whereas the S-ethylsulphoxide (**VI**) predominantly formed the R/S disulphoxide diastereo-isomer (**II**). However, comparison of these results with those from the methylsulphoxide diastereoisomer (**II**) indicated that the S-diastereoisomer (**V**) predominantly formed the S/S disulphoxide diastereoisomer (**II**) whereas the R-diastereoisomer (**V**) also predominantly formed the R/S disulphoxide diastereoisomer (**II**).

The site- and stereoselectivities observed may be explained on the basis of preferred conformations of the C-17 substituents [3,10]. The rate constants for the individual S-oxidations support the observed site- and stereoselectivity observed in the S-oxidative degradation of tipredane in various formulations.

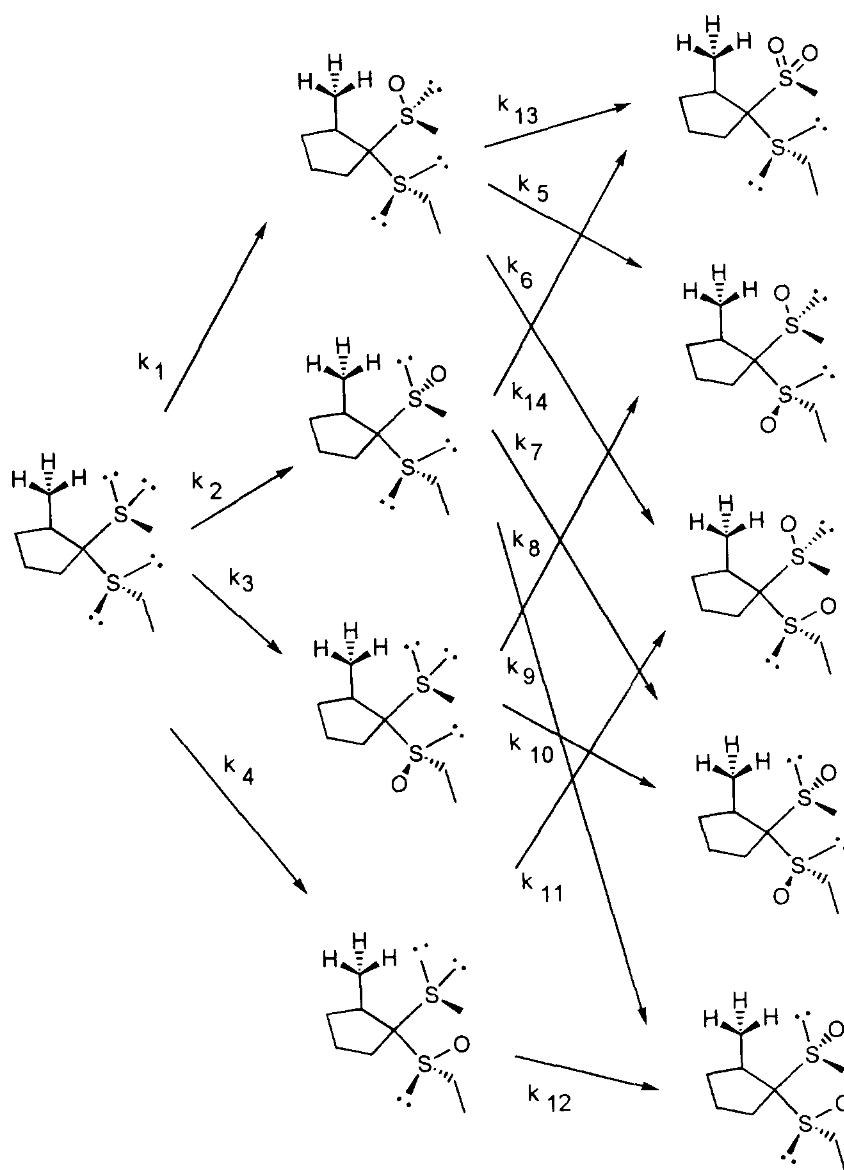

Fig. 8. Identification of the constants for the mono and di S-oxidation of tipredane.

3.7. Stability of the disulphoxide diastereoisomers (II)

3.7.1. Solution stability

The individual disulphoxide diastereoisomers (**II**) were isolated by overloading the 150 mm × 4.6 mm column using the optimised disulphoxide HPLC methodology. The separated fractions had a purity in excess of 99%. The individual diastereoisomeric disulphoxides (**II**) were diluted with mobile phase (methanol–acetonitrile–tetrahydrofuran–pH 7 buffer) to produce fractions of equal concentration which were stored at 40°C for up to 144 h. The results indicated that the disulphoxides underwent degradation via thermolytic elimination of ethylsulphenic acid to the known vinylmethylsulphoxide

Table 1
Values of the rate constants for the mono and di S-oxidation of tipredane

Rate constant	Rate ($l\ mol^{-1}\ min^{-1}$)	Stereochemistry of product	
		MeS(O)	EtS(O)
k_1	200	*S*	–
k_2	50	*R*	–
k_3	9	–	*S*
k_4	70	–	*R*
k_5	5	*S*	*S*
k_6	1	*S*	*R*
k_7	20	*R*	*S*
k_8	3	*R*	*R*
k_9	300	*S*	*S*
k_{10}	700	*R*	*S*
k_{11}	300	*S*	*R*
k_{12}	100	*R*	*R*
k_{13}	1	Sulphone	–
k_{14}	1	Sulphone	–

diastereoisomers (**IX**), i.e. the *R*/*S* and *R*/*R* diastereoisomers degraded to the less polar (based on chromatographic terms) vinylmethylsulphoxide (**IX**), whereas the *S*/*R* and *S*/*S* diastereoisomers degraded to the more polar (based on chromatographic terms) vinylmethylsulphoxide (**IX**). Therefore, the stereochemistry of the vinylmethylsulphoxides (**IX**) could be assigned, i.e. the more polar and less polar diastereoisomers possessed the *S*- and *R*-sulphoxide configurations respectively. The degradation also indicated that only ethylsulphenic acid eliminated, which presumably occurred via a facile syn-periplanar elimation process (see Fig. 9), and that the instability of the disulphoxide diastereoisomers (**II**) was found to mirror that of the corresponding ethylsulphoxide diastereoisomers (**VI**).

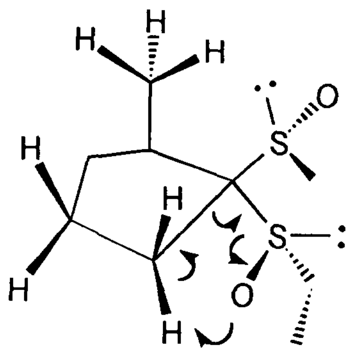

Fig. 9. Three-dimensional representation of the *R*/*S*-disulphoxide diastereoisomer (*R*/*S*-**II**) undergoing facile elimination of ethylsulphenic acid.

Table 2
% Elimination of ethylsulphenic acid from the disulphoxide diastereoisomers (**II**) in ammonium acetate buffer (0.025 M, pH 7.1) in methanol–acetonitrile–tetrahydrofuran–water (16:4:4.8:75.2 v/v) as a function of time at 40°C

Time (h)	% Elimination of ethylsulphenic acid from disulphoxides (**II**)			
	R/*S*	*S*/*R*	*S*/*S*	*R*/*R*
0	0	0	0	0
24	12.6	12.9	6.9	2.5
48	24.7	24.1	13.6	5.5
76	32.4	32.7	18.9	7.7
144	53.7	54.9	34.3	15.0

The rate of elimination appeared to be dependent on the stereochemistry of the diastereoisomers (**II**) in that the rate of elimination was found to be as follows: $R/S \approx S/R > S/S > R/R$ (see Table 2). In contrast, in the solid state (powdered) at 40°C only the *R*/*S* diastereoisomer underwent significant elimination of ethylsulphenic acid. On prolonged heating at 100°C, the *S*/*S* diastereoisomer underwent additional elimination of methylsulphenic acid to generate the corresponding vinylethylsulphoxide (**X**). Therefore, it could be deduced that the more polar vinylethylsulphoxide (expressed in terms of chromatographic performance) possessed the sulphoxide in the *S* configuration.

3.7.2. The effect of solution pH on the stability of the disulphoxide diastereoisomers (II)

The solid disulphoxide diastereoisomers (**II**) were dissolved in pH 2, 7 and 9 buffers and stored at 40°C for 24 h. There appeared to be no difference in the rates at which the individual disulphoxide diastereoisomers degraded in differing pH solution, which is in contrast to the findings for the monosulphoxides [3]. The rate of elimination was found to be as follows: $R/S \approx S/R > S/S > R/R$. The only degradative products observed were elimination products. Therefore, it can be concluded that the elimination of ethyl-

sulphenic acid was independent of pH control for each disulphoxide diastereoisomer.

The relative stability of the diastereoisomeric disulphoxides towards thermal elimination of alkylsulphenic acid can possibly be rationalised in terms of the preferred energy conformations of the disulphoxide rotamers around the C-17 position, certain of which will favour the syn-periplanar elimation process. The preferred rotamer population distribution will be dictated by minimisation of interactions between the two disulphoxide moieties and the steric bulk of the two alkyl groups of the C-17 substituents and the C-18 methyl group. The most unstable disulphoxide diastereoisomer possessing the *R/S* configuration can theoretically minimise these effects with the ethylsulphoxide moiety set up for the concerted α,β elimination of ethylsulphenic acid (see Fig. 9).

4. Conclusions

The disulphoxide diastereoisomers (**II**) have been unambiguously shown to be formed in the presence of excess oxidant in formulations containing excipients with peroxide impurities. The spectroscopic data were in accordance with these previously reported for the disulphoxides which were formed in liver homogenates [4].

The monosulphoxides (**V** and **VI**) showed a high degree of site- and stereoselectivity towards further S-oxidation. S-Oxidation on the C-17 β-substituent of tipredane occurred at a rate approximately 50-fold faster than that on the α-substituent. The observed site- and stereoselectivity of the S-oxidation of the monosulphoxides (**V** and **VI**) mirrored the disulphoxide diastereoisomeric distribution obtained when tipredane was reacted with excess oxidant.

The disulphoxide diastereoisomers (**II**) exhibited differing degrees of stability towards thermolytic elimination of alkylsulphenic acid which was dependent on the stereochemistry of the C-17 substituents. Elimination of ethylsulphenic acid was preferentially favoured.

It is possible that the disulphoxide diastereoisomers (**II**) formed metabolically also undergo nonenzymatic elimination of ethylsulphenic acid to generate the *S*- and *R*-vinylmethylsulphoxides (**IX**). Further oxidation at the sulphur would give the sulphone. Enzymatic oxidation at the 6-position would then yield the observed 6β-hydroxy-16,17-vinylmethyl sulphoxides (**III**) and sulphone (**IV**). This may partially explain the site- and stereoselectivity of the S-oxidation of tipredane that is observed with in-vivo metabolism.

Acknowledgements

The authors thank the following for technical support and advice: Dr. I. G. Beattie (MS), Mr. D. Hunter (NMR), Drs. A. Freer and N. Gensmantel (X-ray crystallography) and the SERC (for awards to D.B.W. and J.A.G.).

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