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THE SYNTHESIS OF LABELLED FORMS OF SAQUINAVIR

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SUMMARY

The development of the HIV-protease inhibitor, saquinavir (Ro 31-8959), required a range of analytical methods for the measurement of the parent drug and drug-related material in biological fluids. This paper describes the synthesis of 14-carbon and tritium labelled compounds used for *in vivo* and *in vitro* investigations of the absorption and disposition of saquinavir in animals and man. It also discusses the preparation of saquinavir labelled with deuterium and stable isotopes of carbon and nitrogen. These forms of the drug were needed for bioequivalence studies in which HPLC/MS/MS was employed for the measurement of plasma concentrations. Finally, the synthesis of a 125-iodine labelled tracer used in a radioimmunoassay for saquinavir is described.

Key words: Saquinavir, AIDS, [^{14}C], [^{3}H], [^{125}I], stable labels.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) results from infection by the human immunodeficiency virus (HIV). It continues to be a major health hazard and antiviral therapies, based on the inhibition of the viral reverse transcriptase, are deficient for reasons of limiting toxicity and drug resistance. This created an urgent need for new effective therapies which would resolve the viral burden in infected individuals and allow the restoration of their immune function. The HIV proteinase catalyses the formation of active enzymes from inactive precursors (polyproteins) and is essential for the production of mature viral particles and hence for the spread of the disease. A number of enzymes, including reverse transcriptase, which are needed for the continuing replication of the virus result from the activity of HIV proteinase. This enzyme is extremely unusual in being able to catalyse the hydrolysis of proteins adjacent to prolyl residues and this property enabled the design of highly specific peptide-like structural mimetics of these substrates.

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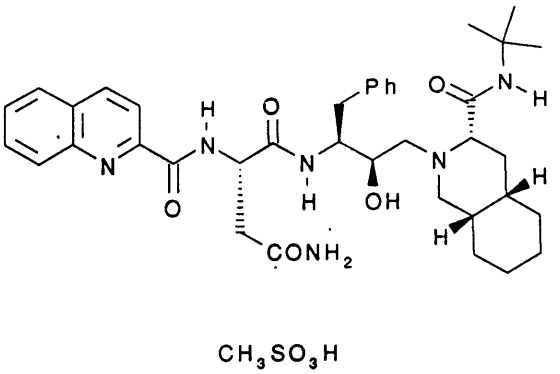
Saquinavir (Figure 1) was the first proteinase inhibitor to be licensed for the treatment of HIV infections and has established itself as a leading component of the multi-drug therapy now used for the disease [1].



CH_3SO_3H

Figure 1 The structure of saquinavir

Various aspects of the development of saquinavir required different labelled forms of the drug and it is the purpose of this paper to describe the synthesis of these compounds. 14-Carbon labelled saquinavir was prepared for standard absorption, distribution, metabolism and excretion experiments in animals and man. Tritiated saquinavir was synthesised for *in vitro* studies which required material of higher specific activity than could be obtained with 14-carbon.

The large inter- and intra-subject variability in plasma concentrations of saquinavir after oral administration meant that it was difficult to carry out classical bioequivalence studies. A deuterated ($^2H_4$ or $^2H_5$) "internal standard" was made, administered to volunteers with the various formulations and plasma levels of both protonated and deuterated saquinavir determined simultaneously by means of HPLC/MS/MS [2]. This reduced the number of subjects needed for these studies. An analytical internal standard ($^{13}C_6$-$^{15}N_1$-$^2H_3$) was also prepared for this assay.

A radioimmunoassay was developed for the routine analysis of HIV-positive samples. The synthesis of the 125-iodine labelled tracer is also discussed in this paper.

RESULTS AND DISCUSSION

Saquinavir has been prepared by various convergent syntheses, e.g. [3], which all involve the preparation of the amino-acids (or amino-acid mimetics) followed by the formation of the amide bonds. The most attractive site for labelling is the quinaldine ring system which contains none of saquinavir's six asymmetric centres and which can be introduced at the final synthetic step.

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14-Carbon labelled saquinavir

14-Carbon labelled quinaldic acid is relatively accessible from universally 14-carbon labelled aniline *via* the Döbner-Miller synthesis (Figure 2). Reaction of [benzene ring-U-^{14}C]-aniline with crotonaldehyde (formed *in situ* from paraldehyde) in the presence of acid [4], followed by dehydration and dehydrogenation gave a 25% yield of quinaldine (1) which was purified chromatographically. Oxidation to [benzene ring-U-^{14}C]-quinaldic acid (3) was carried out by means of a two-stage process, bromination of the 2-methyl group to give the tribromide (2), followed by hydrolysis [5] to the required labelled quinaldic acid (3). After chromatographic purification, a 42% yield (from quinaldine) of quinaldic acid (3) was obtained.

Ro 31-8875 [3] was hydrogenated to remove the benzyloxycarbonyl protecting group and the resultant amine (Ro 32-0445) condensed with the radioactive quinaldic acid (1) using 1-hydroxybenzotriazole or N-hydroxysuccinimide and dicyclohexylcarbodiimide as the coupling agents. After purification by normal phase high performance liquid chromatography and crystallisation as its methane sulphonate salt, saquinavir, with a specific activity of 69μCi/mg and radiochemical purity of >98%, was obtained in a yield of 75%.

Deuterated saquinavir

In order that plasma concentrations of the "labelled" and "unlabelled" forms of saquinavir can be determined simultaneously it is important that the ions monitored in the mass spectrometer are sufficiently far apart that there is no overlap. The drug contains 38 carbon atoms and so the natural abundance of 13-carbon atoms (~1%) means that the "M+1" ion (m/z = 671) is approximately 40% of the size of the protonated molecular ion (m/z = 670). Similarly the "M+2" ion is roughly 15% of the molecular ion and the "M+3" ion about 5% of the parent. Thus an increase in molecular weight of at least 3, and preferably 4, is needed in order to prevent significant interference of the "normal" drug with its heavier derivative. It would be possible to prepare 13-carbon labelled forms of saquinavir, but quantities of the order of 100 g were needed for the planned studies and this would have been prohibitively expensive. A multiply deuterated compound is very much cheaper to prepare, but introduces problems of deuterium-proton exchange which needed to be solved.

Deuterated quinaldic acid is accessible from deuteroaniline using the Döbner-Miller synthesis discussed above. Hexadeuterobenzene is commercially available and nitration and reduction lead to aniline. The nitration of hexadeuterobenzene with concentrated nitric and sulphuric acids proceeded uneventfully to give pentadeuteronitrobenzene in 73% yield after distillation (Figure 3).

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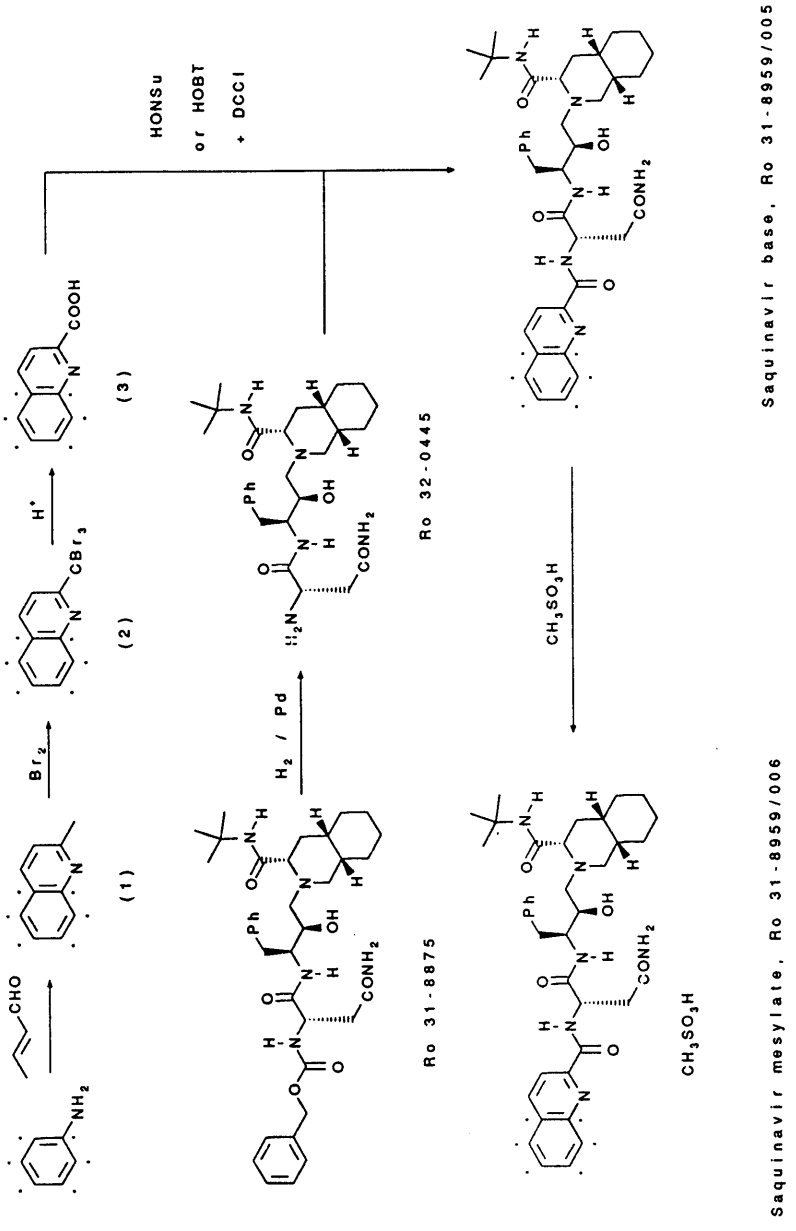

Figure 2 Synthesis of 14-carbon labelled saquinavir (*, 14-carbon atoms)

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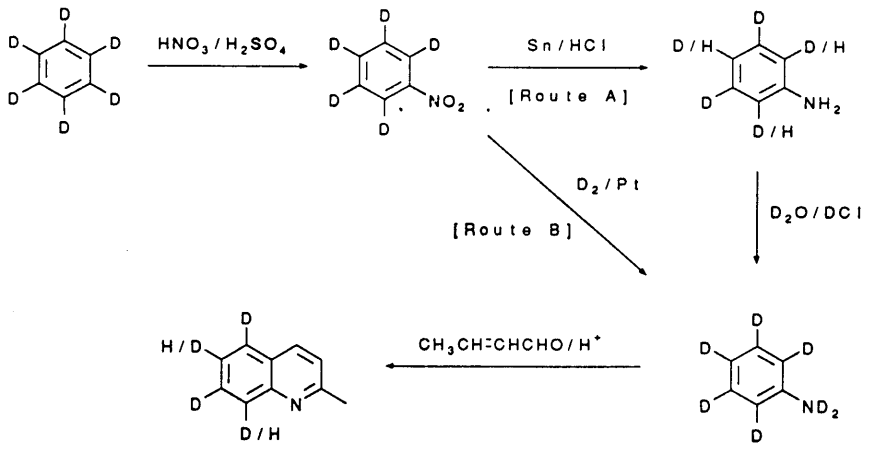

Figure 3 The synthesis of deuterated aniline and its cyclisation to quinaldine

The mass spectrum of the product had intense ions at m/z = 128 (M^+, 90%) and 82 (M^+-NO2, 100%). No significant exchange of protium for deuterium occurred as shown by the very small ion at m/z = 127 (~3%) and the absence of any signals between m/z = 123 and m/z = 126. (The mass spectrum of the starting material, hexadeuterobenzene, showed a similarly small M^+-1 peak).

A number of methods were tried for the reduction of the nitrobenzene. Reduction with tin and concentrated hydrochloric acid (Route A) gave a good chemical yield, but the deuterium atoms ortho and para to the amino group exchanged with the protons of the solvent. The mass spectrum of pure pentadeuteroaniline exhibits a base peak of molecular ion with m/z = 98. The M^+-1 ion has about 14% of the intensity of this. The intensity of the corresponding ion from the aniline prepared by reduction of nitrobenzene was 35% of the molecular ion, which suggests that about 20% of the product contained only 4 deuterium atoms. This was confirmed by the NMR spectrum which showed that both ortho and para deuterium atoms were partially exchanged in the ratio of 2:1, but that the meta protons were almost entirely deuterated.

The elimination of the hydrogen atoms from the aniline was largely accomplished by evaporation from dilute deuterium chloride solution to remove the protons on the nitrogen atom followed by heating under reflux for 18 - 20 hours with more deuterium chloride solution. The NMR spectrum showed that the intensity of the ortho and para protons were reduced by this treatment to comparable amounts to the meta protons.

Subsequently, catalytic reduction of a solution of D_5-nitrobenzene in mono-deuterated methanol over platinum oxide under 0.1 bar pressure of deuterium (Route B) was found to yield D_7-aniline directly. This was converted to

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anilinium chloride by titration with approximately 11M deuterium chloride in deuterated water in 74% yield from D_6-benzene.

Preparation of D_4-quinaldine and Ro 31-8959/020

D_8-Anilinium chloride was heated with a large excess of crotonaldehyde (instead of paraldehyde) in the presence of concentrated hydrochloric acid and a little iodine to give a black mixture from which the basic quinaldine was isolated and purified chromatographically in ~50% yield (Figure 3). Unfortunately the NMR spectrum showed that some loss of deuterium had occurred at the positions originally ortho and para to the nitrogen atom of aniline. The deuterated quinaldine was oxidised to its tribromo-derivative and this hydrolysed to quinaldic acid, without further loss of deuterium, in 43 % yield from quinaldine. The deuterated quinaldic acid was then converted to saquinavir mesylate (Ro 31-8959/020), in 68% yield after recrystallisation, by the same route as the 14-carbon labelled material. The mass spectrum of the product indicated a D_4:D_3:D_2 percentage ratio of 41:33:26.

Preparation of D_5-quinaldine and Ro 31-8959/048

A solution of deuterium chloride in deuterium oxide was substituted for concentrated hydrochloric acid in an attempt to prevent the partial replacement of hydrogen for deuterium atoms during the formation of quinaldine (Route A, Figure 4).

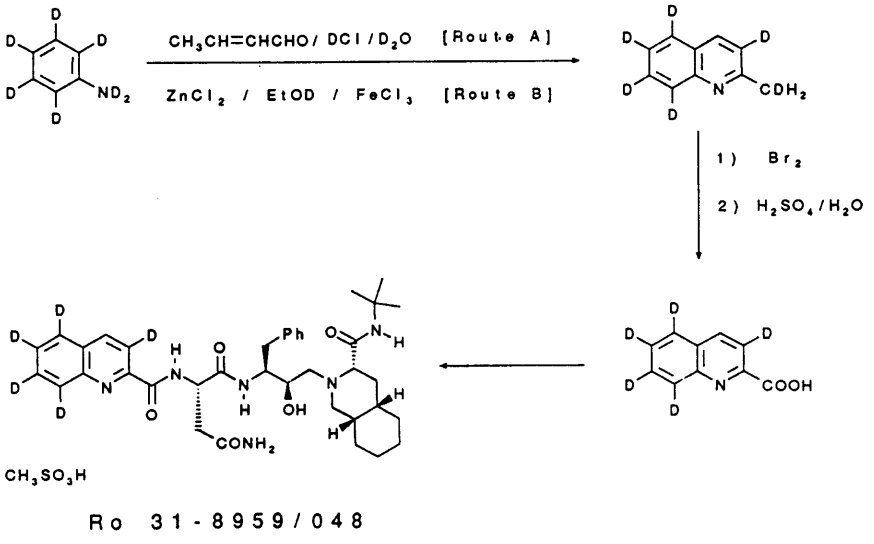

Figure 4 The synthesis of D5- saquinavir (Ro 31-8959/048)

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NMR analysis showed that the product was not the required tetra-deuterated compound but a mixture containing predominantly D_6-quinaldine (Figure 5). Approximately 85% of the atoms attached to C-3 of the quinaldine were deuterium and there was also some substitution of hydrogen by deuterium on the methyl group. On the other hand, about 15% of the deuterium atoms were lost from carbons 6 and 8. These results must have been caused by partial deuterium-hydrogen exchange of the ß and δ protons of the crotonaldehyde (present in large excess) with the solvent.

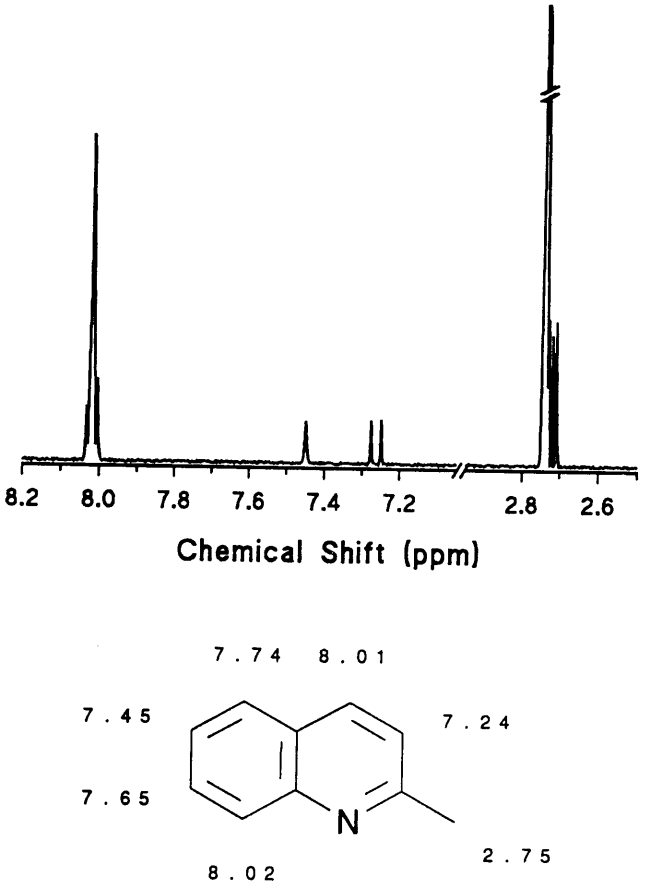

Figure 5 **NMR Spectrum of Quinaldine prepared from D-5 Aniline by Döbner-Miller synthesis using deuterium chloride**

Three methods were tried to reduce the proportion of exchangeable hydrogen atoms; the amount of crotonaldehyde was reduced by 50% and the volume of deuterium chloride was doubled. The isotopic purity was increased in both cases, although the yields were no more than half of those from the original synthesis. As increased acid reduced the

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yield, the Lewis acid, zinc chloride, was substituted for the excess acid with monodeuteroethanol as solvent and ferric chloride as oxidant [6]; this cocktail produced quinaldine in yields of 70%-80% (Route B).

The hexadeuteroquinaldine was transformed into D_5-saquinavir (Ro 31-8959/048) *via* quinaldic acid by the same procedure as the D_4-analogue in yields of up to 56%. The mass spectrum of the product prepared by Route A (Figure 6) indicated a D_6:D_5:D_4:D_3 percentage ratio of 2.8 : 63.3 : 27.3 : 6.6, whereas that prepared by Route B contained a higher proportion of both D_5 and D_6 material (D_6:D_5:D_4 = 5.5 : 72.9 : 21.6).

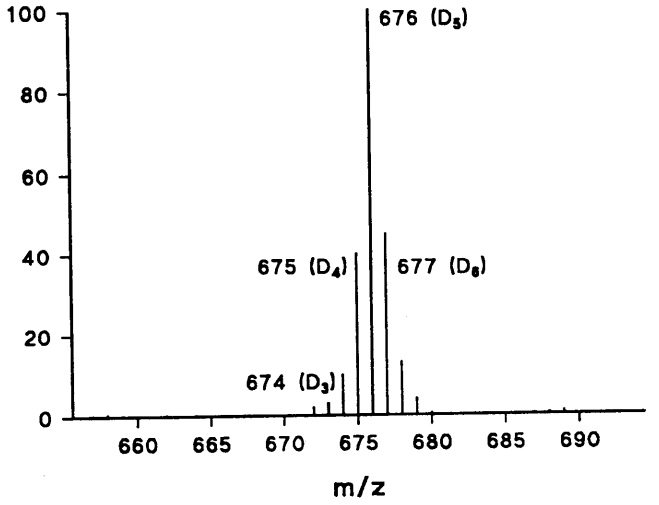

Figure 6 Part of the FAB (thioglycolic acid) Mass Spectrum of Ro 31-8959/048 (Mainly D_5 labelled)

Mass spectral internal standard

In order that measurement of the internal standard for the simultaneous mass spectral assay of D_0- and D_5-saquinavir should not compromised by the presence of the deuterated compound, it was necessary to synthesise a form of saquinavir with a molecular weight at least nine Daltons greater than that of the unlabelled drug. Accordingly $^{13}C_6$-benzene was nitrated with ^{15}N-nitric acid in concentrated sulphuric acid to give nitrobenzene with a molecular weight 7 Daltons greater than that of the "light" compound (Figure 7). This was reduced to aniline, which was cyclised to quinaldine with crotonaldehyde in deuterium chloride solution. The NMR spectrum was complicated by

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coupling between the protons and the 13-carbon atoms, but the mass spectrum indicated that several deuterium atoms had been introduced.

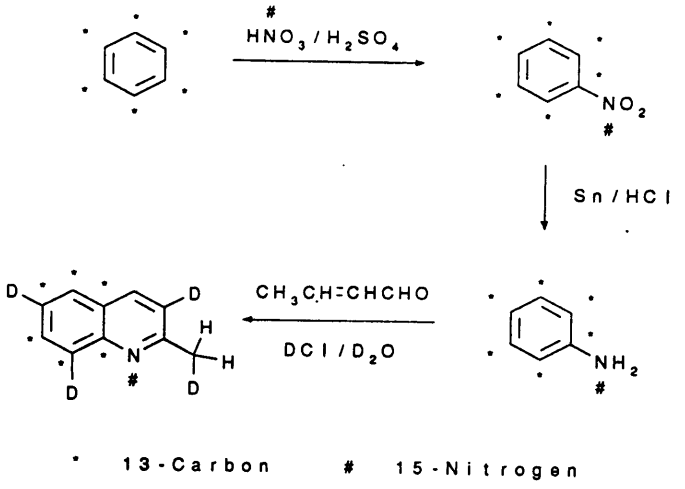

Figure 7 **The Synthesis of 13-C,15-N,2-H-Quinaldine**

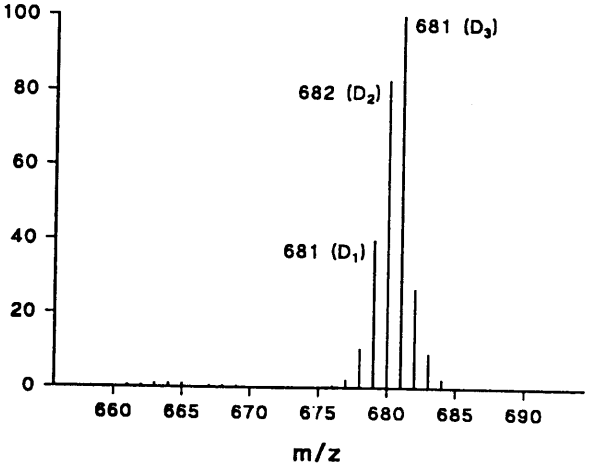

Figure 8 **Part of the FAB (thioglycolic acid) Mass Spectrum of Ro 31-8959/046 (13-C,15-N,2-H-labelled)**

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The "heavy" quinaldine was oxidised to quinaldic acid via tribromoquinaldine and Ro 31-8959/046 prepared by coupling the N-hydroxysuccinimide ester with Ro 32-0445. The mass spectrum of the product (Figure 8) indicated that it contained approximately equal amounts of D_2 and D_3 material.

Tritiated saquinavir

Although it is possible to introduce tritium into the saquinavir molecule with tritiated water in a similar manner to the deuterated compounds, this method is likely to cause exchange with protons and unacceptably low specific activity. A brominated saquinavir analogue was, therefore, prepared and reduced with tritium gas (Figure 9). Para-bromoaniline was cyclised to 6-bromoquinaldine in 71% yield with zinc chloride in the presence of concentrated hydrochloric acid and p-chloranil as oxidising agent [7]. The methyl group of the quinaldine was brominated as with previous analogues and hydrolysed with dilute sulphuric acid to give bromoquinaldic acid. Bromosaquinavir was prepared by coupling the N-hydroxysuccinimide ester of bromoquinaldic acid with the amine, Ro 32-0445, purified chromatographically and tritiated in ethanol over palladised charcoal.

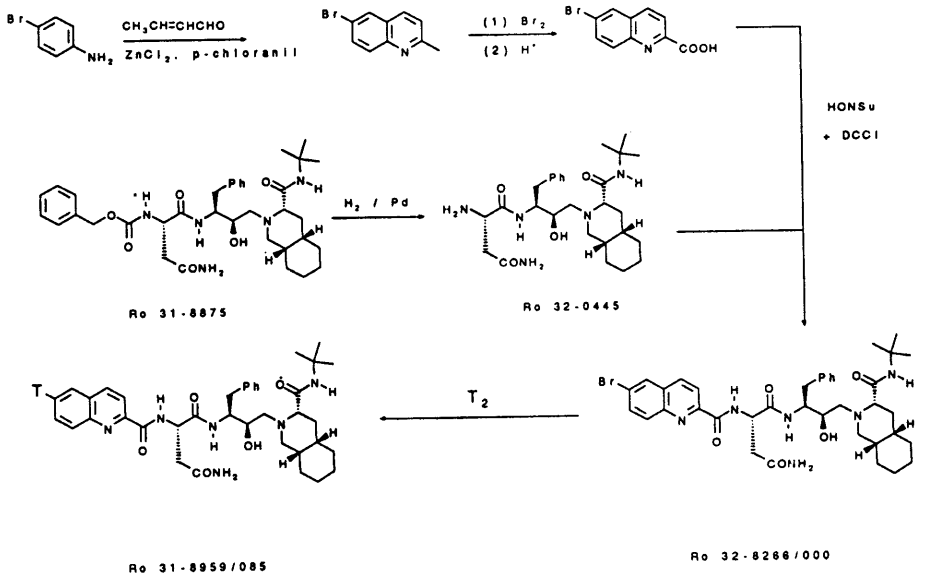

Figure 9 The Synthesis of tritiated saquinavir

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Iodinated analogue of saquinavir

Saquinavir is used for the treatment of a dangerous disease and a simple assay for the determination of plasma levels of the drug in HIV-positive patients was considered important. Since radioimmunoassays require relatively little manipulation by the analyst, they are suitable for such samples. The metabolism of saquinavir has been shown only to involve oxidation of the decahydroisoquinoline ring system and its attached tertiarybutyl group [8]. It was, therefore, important for the specificity of the immunoassay that antibodies should be raised to this part of the molecule in order to minimise cross-reactivity with metabolites. The linkage to both the immunogenic protein and to the 125-iodine labelled group of the tracer was, consequently, made at the opposite end of the molecule by replacement of the quinaldic acid with a suitable group.

3-(4-Hydroxyphenyl)propionic acid (Figure 10, 1) was condensed with the amine (Ro 32-0445, prepared from Ro 31-8875 as discussed above) in the presence of N-hydroxybenzotriazole and dicyclohexylcarbodiimide. After normal phase HPLC purification a yield of approximately 60% of the amide, Ro 32-2446 was obtained. This was iodinated with chloramine-T and 1 mCi of sodium 125-iodide to give, after reverse phase HPLC purification, 206 μCi of the required tracer.

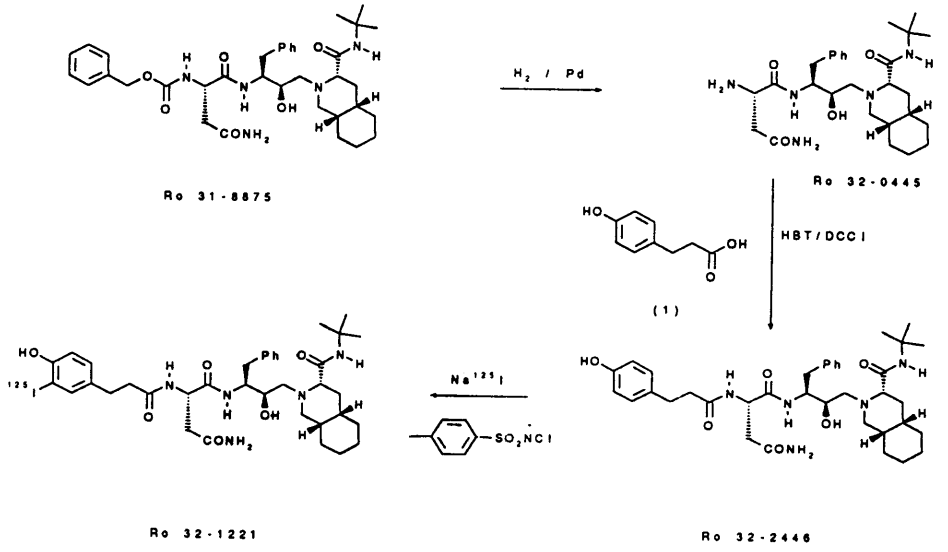

Figure 10 The synthesis of the 125-iodine labelled tracer for saquinavir radioimmunoassay

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EXPERIMENTAL

[Benzene ring-U-^{14}C] labelled quinaldic acid was prepared from [benzene ring-U-^{14}C] labelled aniline by Amersham International plc as described below.

Ro 31-8875 was prepared essentially by the method described in British Patent Application No. 8927913.7 in the Kilo laboratory of Hoffmann-La Roche, Basle, Switzerland.

All tetrahydrofuran used was redistilled from calcium hydride just prior to use and organic solutions were dried over anhydrous magnesium sulphate before evaporation at reduced pressure, unless otherwise stated.

[U-^{14}C]-Quinaldine (Figure 2, 1)

Benzene ring-U-^{14}C]-aniline hydrochloride (242mCi, 2.02mmol) was dissolved in concentrated hydrochloric acid (0.4ml, 4mmol) and cooled to 0°C. Paraldehyde (0.8ml, 6mmol) was added and the mixture stirred at room temperature under a nitrogen atmosphere for 1.5 hours before heating under reflux for 3 hours. After cooling, the mixture was made basic with sodium hydroxide solution and the product extracted into dichloromethane. The extracts were washed with brine, dried and evaporated to afford a dark oil.

Acetic anhydride (0.2ml, 2.1mmol) was added and the mixture heated under reflux for 20 minutes. After cooling, the product was extracted into dichloromethane. The extracts were washed with sodium bicarbonate solution and with brine and dried. After evaporation of the solvent the crude material was purified by chromatography on silica gel using 1:6 ethyl acetate:hexane to afford pure quinaldine (60mCi, 25%).

α,α,α-Tribromo-[benzene ring-U-^{14}C]-quinaldine (Figure 2, 2)

The above quinaldine (60mCi, 0.5mol) was heated to 80°C with sodium acetate (0.25g, 3.05mmol) in glacial acetic acid (0.66ml). A solution of bromine (80μl, 1.6mmol) in glacial acetic acid (0.66ml) was added and the mixture heated to 120°C until sodium bromide precipitated out. After standing at 100°C for one hour, the reaction mixture was cooled to room temperature, made basic with aqueous sodium carbonate and extracted into dichloromethane. The organic solution was washed with brine, dried and evaporated to dryness. The crude material was purified by chromatography on silica gel, eluting first with hexane and then 8:1 hexane:dichloromethane. The yield of pure product was 36.9mCi (61%).

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[Benzene ring-U-^{14}C]-quinaldic acid (Figure 2, 3)

α,α,α-Tribromo-[benzene ring-U-^{14}C]-quinaldine (59.6mCi, 0.68mmol) was heated to 110°C with 10% sulphuric acid (1.5ml) for 18 hours. A further 1.5ml of the acid was added and the reaction continued for another 4 hours. After basification with aqueous sodium bicarbonate, the solution was washed with dichloromethane and the pH reduced to 3 with dilute acid. The product was extracted into dichloromethane which was washed with brine and dried. The solvent was removed, radio-inactive quinaldic acid added and the product purified by gradient reverse phase HPLC using a Dynamax ODS column (Solvent A : 1% acetic acid in water, Solvent B : acetonitrile, Gradient : 5% B for 5 minutes, then 5-90% B over 25 minutes.)

The main product-containing fractions were combined to give pure quinaldic acid (40.7mCi, 68%) with a specific activity of 278μCi/mg. Purity was demonstrated by both reverse phase HPLC and TLC. The HPLC system used a Hypersil 5μ ODS column, the above solvents and a gradient of 5-100% B at 1ml/min over 10 minutes and showed a purity of >98%. TLC on silica gel using a solvent system of 12:5:3 butanol : water : acetic acid gave a radiochemical purity of 99%.

2-[3(S)-[(L-Asparaginyl)-amino]-2(R)-hydroxy-4-phenylbutyl]-N-tert-butyl-decahydro(4aS,8aS) -isoquinoline-3(S)-carboxamide (Figure 2, Ro 32-0445)

Ro 31-8875 (300mg:0.45mM) in chloroform (45ml) was washed once with saturated sodium bicarbonate solution (15ml) to remove any hydrogen chloride present. The chloroform solution was dried and evaporated to dryness. No loss of weight was observed. The residual solid was hydrogenated for three and a half hours in ethanol (15ml) over 10% palladium on carbon (ca. 50mg). The catalyst was then removed by filtration. Evaporation of the filtrate gave a quantitative yield of the product. TLC showed a single spot with Rf=0.25 (25% ethanol in dichloromethane).

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[benzene ring-U-^{14}C]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide (Ro 31-8959/005, saquinavir).

[Benzene ring-U-^{14}C] labelled quinaldic acid (60 mg: 0.34 mmole: 16.68mCi) in dry tetrahydrofuran (9ml) was added to the above amine (240 mg:0.45 mmole) under an atmosphere of argon. The solution was stirred and cooled to 0°C before the addition of 1-hydroxybenzotriazole (60mg: 0.45mM) and N-ethylmorpholine (60μl:0.48mM) in dry tetrahydrofuran (0.6ml). Dicyclohexylcarbodiimide (93mg:0.45mM) in dry tetrahydrofuran (0.6ml) was then added

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and the mixture stirred at 0°C before standing at 4°C for 64 hours. Dicyclohexylurea was removed by filtration and was washed with tetrahydrofuran (5ml). Evaporation of the filtrate gave a gum which was dissolved in ethyl acetate (15ml) and washed with saturated sodium bicarbonate solution (5ml). Drying and evaporation of the solvent left a gum which was purified by normal phase high performance liquid chromatography. (Column Magnum partisil, eluent 20% isopropanol in dichloromethane, flow rate 2.8 ml/min, detection UV at 280 nm, retention 12 minutes. The product-containing fractions were combined and evaporation gave a yield of 173mg (12 mCi:75%).

{Mass spectrum : m/z 671 (93%, $M+H^+$); 672 (46%); 149 (38%); 138 (100%); 128 (36%); 150 (35%), TLC-radioscan : silica plate developed with 25% ethanol in dichloromethane : single spot Rf=0.75, Analytical HPLC, column : Whatman partisil ODS-3 250 x 6.25 mm, eluent : gradient from 20% acetonitrile in 1% triethylammonium phosphate to 50% acetonitrile over 30 minutes, flow rate: 1ml/minute, detector : Reeve radiochemical detector and LKB 2140 rapid scanning ultraviolet detector, retention : 24 minutes. The radiochemical detector showed a 1% impurity (more polar), but there were no obvious impurities in the UV trace.

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[benzene ring-U-^{14}C]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide methane sulphonate Figure 2, Ro 31-8959/006, saquinavir mesylate).

Unlabelled Ro 31-8959/006 (789mg, 1.03mmol) was partitioned between ethyl acetate (20ml) and 10% aqueous potassium carbonate (10ml). The organic layer was washed twice with aqueous potassium carbonate (10ml), dried over anhydrous potassium carbonate and evaporated to give a white foam. Radioactive Ro 31-8959/005 (196mg, 1.931mCi) was dissolved in isopropanol (6.5ml) with the above foam (648mg, 0.965mmol) and treated with a solution of methane sulphonic acid (121mg, 1.26mmol) in isopropanol (1.2ml). The solution was stirred and allowed to stand at 4°C for 2 hours before the white solid was filtered off, washed with isopropanol (2ml), ether (5ml) and dried. Yield 822mg (1.89mCi, 98%, 2.3μCi/mg). $C_{38}H_{50}N_6O_5.CH_4O_3S$ requires C 61.08; H 7.10; N 10.96, found C 60.39; H 7.24; N 10.75; H_2O 1.5, water-free C 61.32; H 7.18; N 10.92. (TLC-radioscan : silica plate developed with 120:20:3:2 chloroform : methanol : acetic acid : water, >98% purity).

Pentadeuteronitrobenzene (Figure 3)

Concentrated nitric acid (60 ml, 0.85 mole) was mixed with concentrated sulphuric acid (68 ml) keeping the temperature below 50°C. Hexadeuterobenzene (50 ml, 44 g, 0.56 mole) was then added at a temperature of 30°C - 55°C. The reaction mixture was then stirred at 60°C for 45 minutes and poured onto ice. When the ice had melted,

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the aqueous layer was discarded and the nitrobenzene washed with water (50 ml) and dried over anhydrous calcium chloride (~5 g). The total crude yield from four similar preparations was 94%. The combined samples (246 g) were distilled under reduced pressure (water-pump) to give 189.7 g (73%) yield of pure material. With an additional extraction step prior to distillation, the yield could be increased to 87%.

Heptadeuteroaniline (Figure 3, Route A)

Concentrated hydrochloric acid (300 ml) was added in 5 ml portions to a mixture of granulated tin (90 g) and pentadeuteronitrobenzene (30 g), swirling and cooling the reaction mixture after each addition. After heating on a boiling water bath for 1 hour, the solution was basified with 5 N aqueous sodium hydroxide (750 ml). The precipitated stannous hydroxide was removed by filtration and washed with ether (3 x 150 ml). The ethereal solution was removed and the aqueous layer extracted with more ether (2 x 150 ml). After drying over anhydrous potassium carbonate, the organic solution was evaporated to give aniline of good purity (16.8 g, 73%).

Heptadeuteroaniline and anilinium hydrochloride (Figure 3, Route B)

A solution of D_5 nitrobenzene (384.4 g) in D_1 methanol (300 ml) was stirred under nitrogen before the addition of platinum oxide catalyst (7.7 g). The stirring was continued as the reaction vessel was evacuated, refilled with nitrogen, re-evacuated and filled with deuterium gas. The mixture was stirred at 400 rpm until the catalyst had been reduced and was then increased to 800 rpm until uptake of deuterium had ceased after 4.5 hours and the solution had become virtually colourless. The catalyst was removed by filtration under argon, washed with methanol (84 ml) and evaporated to dryness to give 283.5 g (94%) of a golden oil. The crude aniline was distilled under reduced pressure (0.01 bar) at 70-72°C to give 270.3 g (90%) of a colourless oil.

The deuterochloride salt was prepared by emulsification of D_7 aniline (260 g) in deuterated water (115 g) at 5°C under argon and dropwise addition of 35% deuterium chloride (234 ml, 1 equivalent). The mixture was frozen with liquid nitrogen, the pressure gradually reduced and the deuterated water removed by distillation at 0.04 mbar overnight. The colourless crystalline residue was dried under vacuum at 42-45°C to give 341.2 g of colourless crystals (94.7%).

α,5,6,7,8-Pentadeuteroquinaldine (Figure 3)

Pentadeuteroaniline (34 g) was dissolved by the slow addition of concentrated hydrochloric acid (140 ml, cooling with an ice bath) over a period of 10 minutes. Crotonaldehyde (70 ml) was added dropwise over 10 minutes,

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followed by iodine (3.5 g) and the mixture was heated at 100°C for 1 hour under an atmosphere of argon. The reaction mixture was cooled to 80°C, treated with a further 70 ml of crotonaldehyde and heated for another hour at 100°C. The cooled black mixture was added to a ice (~400 g) and 5 N aqueous sodium hydroxide (250 ml). The solid residue in the flask was extracted three times with 1:1 sodium hydroxide : dichloromethane (100 ml) and the mixed solvents added to the melted ice. The layers were separated and the aqueous solution extracted twice more with dichloromethane (200 ml). The combined organic layers were washed with water and dried over anhydrous potassium carbonate, filtered and evaporated to a black tar.

This tar was dissolved in warm acetic anhydride (135 ml), boiled for 20 minutes, cooled and evaporated to dryness. The dark residue was partitioned between dichloromethane (350 ml) and 2N sulphuric acid (180 ml) and the organic layer re-extracted with more sulphuric acid (4 x 180 ml). The combined acid solutions were washed twice with dichloromethane and basified by the cautious addition of 5N aqueous sodium hydroxide (400 ml). The crude quinaldine was extracted from the alkaline solution with dichloromethane (5 x 120 ml), dried over anhydrous potassium carbonate, filtered and evaporated to a dark oil (32.5 g). Purification was effected by chromatography on a silica column (300 g), eluting the compound (28.5 g, 55%) with 10% ethyl acetate in hexane (500 - 1800 ml).

α,α,α -Tribromo-5,6,7,8-Tetradeuteroquinaldine (c.f. Figure 2)

The above quinaldine (44.5 g) was heated to 70°C with sodium acetate (150 g) in glacial acetic acid (450 ml). A solution of bromine (150 g) in glacial acetic acid (150 ml) was added dropwise over ~1.5 hours so that the temperature was maintained between 70°C and 75°C. The suspension was heated at 120°C for one hour, cooled to room temperature and added to iced water (1.5 l). The solid (89.7 g, 77%) was filtered off, washed with water and dried.

5,6,7,8-Tetradeuteroquinaldic acid (Figure 3)

α,α,α-Tribromo-5,6,7,8-tetradeuteroquinaldine (48.3 g) was stirred and heated under reflux in 2N aqueous sulphuric acid (210 ml) for 10 hours. The hot solution was filtered through "Hyflo" and cooled to 4°C. After standing for several hours, the crystalline hydrobromide salt was isolated by filtration. The salt was dissolved in hot water (100 ml) and heated with active charcoal (~1.5 g) for 15 minutes and filtered through "Hyflo". The filtrate was brought to pH3.5 with saturated sodium bicarbonate solution, extracted with a 1:1 mixture of dichloromethane and tetrahydrofuran (3 x 100 ml), dried over magnesium sulphate, filtered and evaporated to dryness to give 11.5 g of a pale brown solid.

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The filtrate from the crystallisation of the hydrobromide was heated at 80°C with active charcoal (~2 g) for 15 minutes, filtered through "Hyflo" and the pH raised to 3.5 by the addition of saturated aqueous sodium bicarbonate. The product was extracted into dichloromethane (5 x 100 ml) which was dried over magnesium sulphate, filtered and evaporated to a mauve solid (6.5 g). Further extraction of the filtrate with 1:1 dichloromethane : tetrahydrofuran yielded an additional 1.9 g of quinaldic acid. The three samples of quinaldic acid were combined and recrystallised from ethyl acetate (180 ml) to give the pure compound (12.03 g) in 55% yield.

5,6,7,8-Tetradeuteroquinaldic acid-hydroxysuccinimide ester (c.f. Figure 2)

Deuterated quinaldic acid (21.2 g) was stirred with N-hydroxysuccinimide (13.8 g) in dichloromethane (300 ml) at room temperature for 30 minutes until most of the reactants were in solution. The mixture was cooled to 10°C and treated with a solution of dicyclohexylcarbodiimide (27.2 g) in dichloromethane (120 ml) such that the temperature was maintained at between 10°C and 15°C. After this addition (~15 minutes), the suspension was stirred for one hour and allowed to warm up to room temperature. The precipitated dicyclohexylurea was filtered off and washed well with dichloromethane (240 ml). The filtrate was evaporated to dryness and the solid triturated with methyl-tertiarybutyl ether (240 ml). The red solid (30.2 g) was collected by filtration.

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[5,6,7,8-tetradeutero]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide methane sulphonate (Ro 31-8959/020)

Ro 32-0445 (58.1 g, prepared by hydrogenation of Ro 31-8875 as described above) was stirred in tetrahydrofuran under argon until dissolved and treated with quinaldic acid-hydroxysuccinimide ester (29.3 g). The solution was stirred at room temperature for one hour and allowed to stand overnight. The red solution was evaporated to a foam which was dissolved in ethyl acetate (350 ml) and washed with 10% aqueous sodium carbonate (2 x 350 ml) and water (250 ml). The organic solution was dried over magnesium sulphate, filtered and evaporated to dryness. The residue was dissolved in 1,2-dimethoxyethane (350 ml) at 80°C and treated dropwise with methane sulphonic acid (10.27 g) in more dimethoxyethane (35 ml), keeping the temperature between 80°C and 85°C. The suspension was cooled to room temperature and the salt filtered off and washed with dimethoxyethane (2 x 75 ml). The product was recrystallised from methanol and a second crop obtained from the mother liquors. The combined samples were recrystallised from methanol to give 55.6 g (67%) of the required material of good purity. The mass spectrum indicated that the D_4:D_3:D_2 percentage ratio was 41:33:26.

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α,3,5,6,7,8-Hexadeuteroquinaldine (Figure 4, Route B)

A stirred mixture of ferric chloride (243 g), zinc chloride (9.1 g), D_8-anilinium chloride (86g), D_1-ethanol (450 ml, 95%), 37% deuterium chloride solution and deuterium oxide (97 ml) was warmed to 60°C and treated dropwise with crotonaldehyde (35 g), keeping the temperature between 60 and 65°C. The reaction mixture was then heated under reflux for two hours and allowed to stand overnight. Water (250 ml) was added and the mixture basified to pH 11 by the addition of aqueous sodium hydroxide (620 ml, 28%). The ethanol was removed by distillation at 15 mbar and the product isolated by steam-distillation (~ 6 litres of distillate). The quinaldine was extracted with dichloromethane (2 x 500 ml and 1 x 300 ml for 2000 ml of distillate) and the combined organic solutions dried over sodium sulphate, filtered and evaporated to give a colourless oil (59.9 g, 80.3%). This oil (102.2 g) was heated under reflux with acetic anhydride (300 ml) for 20 minutes and the mixture evaporated to dryness. The dark residue was cooled, diluted with dichloromethane (500 ml) and extracted with 10% sulphuric acid (5 x 300 ml). The combined aqueous solutions were then washed with dichloromethane (3 x 200 ml), basified with aqueous sodium hydroxide (~ 400 ml) before the quinaldine was re-extracted with more dichloromethane (5 x 300 ml). The combined organic solutions were dried over sodium sulphate, filtered and evaporated to dryness to give a yellow oil (72.5 g, 70.9%).

α,α,α-Tribromo-3,5,6,7,8-Pentadeuteroquinaldine (c.f. Figure 2)

The above quinaldine (35 g) was heated to 70°C with sodium acetate (122.4 g) in glacial acetic acid (245 g). Bromine (117.5 g) was added dropwise over ~0.5 hours so that the temperature was maintained between 70°C and 80°C. The suspension was heated under reflux for 15 minutes, cooled to 40°C and added to iced water (750 ml). The solid (83.4 g, 92%) was filtered off, washed with water and dried.

3,5,6,7,8-Pentadeuteroquinaldic acid (Figure 4)

α,α,α-Tribromo-3,5,6,7,8-tetradeuteroquinaldine (80 g) was stirred and heated under reflux in 10% aqueous sulphuric acid (550 ml) for 5.5 hours. The hot solution was filtered through "Hyflo" and cooled to 4°C. After standing for several hours, the brown crystalline hydrobromide salt (37.5 g, 70%) was isolated by filtration and washed with acetone (150 ml). The hydrobromide salt (37 g) was dissolved in water (150 ml) and treated with portions of solid sodium bicarbonate (12 g total) to give a solution with a pH of 3. The solution was saturated with solid sodium chloride and extracted with tetrahydrofuran [200 ml (some solid was filtered off and dissolved in 1:1 methanol:tetrahydrofuran) and 2 x 100 ml]. The organic solution was dried over magnesium sulphate, filtered,

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combined with the above methanolic solution and evaporated to a brown solid. This was crystallised from ethyl acetate (300 ml), filtering and then concentrating the hot solution to 200 ml, to give the pure compound (16.1 g) in 63% yield. A second crop of 2.44 g (10%) was obtained from the mother liquors.

3,5,6,7,8-Pentadeuteroquinaldic acid-hydroxysuccinimide ester (c.f. Figure 2)

Deuterated quinaldic acid (24.0 g) was stirred with N-hydroxysuccinimide (15.5 g) in dichloromethane (200 ml) at room temperature for 30 minutes until most of the reactants were in solution. The mixture was cooled to 10°C and treated with a solution of dicyclohexylcarbodiimide (30.6 g) in dichloromethane (100 ml) such that the temperature was maintained at between 10 ± 2 °C. After this addition (~30 minutes), the suspension was stirred for one hour at room temperature. The precipitated dicyclohexylurea (28.2 g, 93%) was filtered off and washed well with dichloromethane (3 x 200 ml). The filtrate was evaporated to dryness and the solid triturated with methyl-tertiarybutyl ether (150 ml). The brown solid (37.25 g, 100% but containing ~3.4% of dicyclohexylurea) was collected by filtration.

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[3,5,6,7,8-pentadeutero]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide methane sulphonate (Ro 31-8959/048, Figure 4)

2-[3(S)-[(L-Asparaginyl)-amino]-2(R)-hydroxy-4-phenylbutyl]-N-tert-butyl-decahydro(4aS,8aS)-iso-quinoline-3(S)-carboxamide (69.29 g, prepared as already described) was stirred in tetrahydrofuran (400 ml) until dissolved and treated with quinaldic acid-hydroxysuccinimide ester (37.0 g) and hydrated hydroxybenzotriazole (1 equivalent). The solution was stirred at room temperature for one hour and partitioned between ethyl acetate (400 ml) and saturated aqueous sodium carbonate solution (400 ml). The organic solution was washed with water, brine, dried over magnesium sulphate, filtered and evaporated to dryness. The residual brown foam was dissolved in 1,2-dimethoxyethane (400 ml) at reflux and treated dropwise with methane sulphonic acid (12.92 g) in more dimethoxyethane (75 ml). The suspension was cooled to room temperature and the salt filtered off (83.95 g, 81%) and washed with dimethoxyethane. The product was recrystallised from methanol (1328 ml, concentrating the hot solution to about 420 ml) and washed with more methanol (150 ml) to give 66.75 g, 64%) of a white solid. Two further crops (23.4 g, 22%) were obtained from the mother liquors. The mass spectrum (Figure 6) indicated that the D_5:D_4:D_3 percentage ratio was 63.7:28.7:7.6.

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$^{15}N_1$-$^{13}C_6$-Nitrobenzene (Figure 7)

^{15}N-nitric acid (1.1 ml, 40% aqueous solution) was treated with concentrated sulphuric acid (1.3 ml) with cooling. U-^{13}C-Benzene (1 g) was dripped in keeping the solution below 25°C. The mixture was stirred vigorously, slowly heated to 60°C, kept at this temperature for 45 minutes before being added to ice (~20 g). The flask was washed out with diethyl ether (10 ml) and these washings used to extract the aqueous mixture. The aqueous solution was re-extracted with ether (3 x 5 ml) and the combined organic solutions dried over anhydrous calcium chloride, filtered and evaporated to give 0.82 g (51%) of the required nitrobenzene with no detectable dinitro compound.

$^{15}N_1$-$^{13}C_6$-Aniline (Figure 7)

The above nitrobenzene (0.82 g) was treated with tin pellets (2.5 g) and the mixture stirred with concentrated hydrochloric acid (8.2 ml, added in 2 ml portions). When the addition was complete, the reaction was heated to 100°C for one hour, cooled and basified with 5 N aqueous sodium hydroxide. Tin salts were filtered off and extracted with dichloromethane (20 ml). The organic layer from the filtrate was separated and the aqueous solution extracted with more dichloromethane (4 x 10 ml). The combined organic extracts were dried over anhydrous potassium carbonate, filtered and evaporated to a colourless oil (0.57 g, 90% yield). The mass spectrum showed the expected base peak with m/z = 100.

$^{15}N_1$-$^{13}C_6$-$^{2}H_4$-Quinaldine (Figure 7)

The above aniline (0.57 g) was dissolved in a mixture of deuterium oxide (2 ml) and deuterium chloride (0.75 ml, 38% solution in deuterium chloride) and the solution evaporated to dryness at 40°C. The residue was redissolved in deuterium oxide (2 ml), again evaporated to dryness and dissolved in deuterium chloride solution (3 ml). The solid residue was stirred with deuterium chloride solution (3 ml) and crotonaldehyde (1.15 ml) was dripped in. Iodine (75 mg) was added and the mixture heated at 110 °C for 1 hour under an atmosphere of argon. The reaction was cooled to 40 °C, more crotonaldehyde (1.15 ml) added and the reaction continued at 110°C for a further hour. After cooling, the black mixture was poured onto ice (~10 g) and 5 N aqueous sodium hydroxide (7 ml). The flask was washed out with a mixture of sodium hydroxide solution and dichloromethane (3 x 2 ml each). The washings were added to the melted ice and the layers were separated. The aqueous solution was extracted with more dichloromethane (2 x 4 ml) and the combined organic extracts dried with anhydrous potassium carbonate, filtered and evaporated to a black oil. This oil was dissolved in acetic anhydride (5 ml), boiled for 20 minutes, cooled and evaporated to dryness. The dark residue was partitioned between dichloromethane (3 ml) and 2N sulphuric acid (3 ml) and the organic layer re-extracted with more sulphuric acid (2 x 3 ml). The combined acidic solutions were

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washed twice with dichloromethane (2 ml) and basified by the cautious addition of 5N aqueous sodium hydroxide. The crude quinaldine was extracted from the alkaline solution with dichloromethane (3 x 3 ml) and the combined organic solutions dried over anhydrous potassium carbonate, filtered and evaporated to a dark oil (0.34 g). Purification was effected by chromatography on a silica column (5 g), eluting the compound (0.24 g, 27%) with 10% ethyl acetate in hexane (30 - 55 ml).

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[$^{15}N_1$-$^{13}C_6$-$^{2}H_3$-]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide methane sulphonate (Ro 31-8959/046, c.f. Figure 2)

This compound was prepared from the above $^{15}N_1$-$^{13}C_6$-$^{2}H_4$-quinaldine and Ro 31-8875 in an analogous manner to the 14-carbon labelled compound in 60% yield after recrystallisation. The mass spectrum (Figure 8) indicated that the D_3:D_2:D_1:D_0 percentage ratio was 38:37:19:6.

6-Bromo-2-quinaldine (Figure 9)

4-Bromoaniline (3.44 g), concentrated hydrochloric acid (5 ml) and para-chloranil (4.9 g) were heated in n-butanol (5 ml) to reflux. A solution of crotonaldehyde (2 ml) in butanol (2 ml) was then added dropwise. After the reaction had been heated under reflux for 20 minutes zinc chloride (2.74 g) in tetrahydrofuran (40 ml) was added in small portions. The reaction mixture was heated under reflux for a further 10 minutes and cooled to 0°C. The solid (4.68g) was filtered off and washed with tetrahydrofuran (30 ml), 2-propanol (3 x 20 ml) and ether (3 x 20 ml).
The zinc complex was triturated with water (80 ml) and concentrated ammonia (80 ml) to give a pale brown solid which was extracted with ether (160 ml, 2 x 80 ml). The organic solution was dried over anhydrous potassium carbonate, filtered and evaporated to give a solid (3.14 g, 71%).

6-Bromo-2-quinaldic acid (Figure 9)

The above bromoquinaldine (2.94 g) was dissolved in acetic acid (29 ml) and heated at 75°C under nitrogen with sodium acetate (6.1 g) for 10 minutes. A solution of bromine (2.2 ml) in acetic acid (6 ml) was then dripped in. After 2 hours heating under reflux at 120°C, the reaction mixture was allowed to cool, poured onto ice (100g) and stood overnight. The precipitate of tetrabromoquinaldine (5.12 g, 84%) was filtered off, washed with water (30 ml) and dried.

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The tetrabromoquinaldine (5.1 g) was heated under reflux with 2N sulphuric acid (40 ml) for 16 hours. As starting material was still present, tetrahydrofuran (20 ml) was added and the heating continued for a further 6 hours. The mixture was cooled and the organic solvent removed by rotary evaporation. The insoluble product (0.575 g, 21%) was filtered off and dried. The filtrate was washed with ethyl acetate and its pH adjusted to 3.5 with saturated sodium bicarbonate. The rest of the unchanged starting material was extracted with ethyl acetate and the combined organic solutions dried over magnesium sulphate, filtered and evaporated to dryness. The residue was reheated with 2N sulphuric acid (50 ml) for 16 hours and a further 0.85 g (30%) of the required product precipitated on cooling.

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[6-bromo]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide (Ro 32-8266/000 Figure 9)

The above bromoquinaldic acid (1.3 g) was suspended in dry dichloromethane (30 ml) with N-hydroxysuccinimide (600 mg) and stirred at room temperature for 30 minutes. The suspension was cooled to 10°C and treated with a solution of dicyclohexylcarbodiimide (1.15 g) in dry dichloromethane (10 ml). The suspension was allowed to reach room temperature and stirred for 4 hours. The dicyclohexylurea was filtered off, the filtrate evaporated to dryness and the residue triturated with ethyl acetate to give 870 mg (48%) of product. A further 1.2 g of impure material was isolated from the mother liquors.

2-[3(S)-[(L-Asparaginyl)-amino]-2(R)-hydroxy-4-phenylbutyl]-N-tert-butyl-decahydro(4aS,8aS)-isoquinoline-3(S)-carboxamide (1.27 g, prepared as already described) was stirred in dry tetrahydrofuran (7 ml) until dissolved and treated with 6-bromoquinaldic acid-hydroxysuccinimide ester (0.86 g). The solution was stirred at room temperature overnight and evaporated to a pale brown waxy solid. This was extracted with ethyl acetate and the solution washed with 10% aqueous potassium carbonate (2 x 20 ml), water (2 x 20 ml), dried over magnesium sulphate, filtered and evaporated to dryness (1.2 g). The crude product was purified chromatographically on silica gel, eluting with 10% ethanol in dichloromethane, to give 0.73 g (39%) of the required compound.

N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-4-phenyl-3(S)-[{N-(2-[6-tritio]-quinolylcarbonyl)-L-asparaginyl}amino]butyl]-(4aS,8aS)-isoquinoline-3-carboxamide methane sulphonate (Ro 31-8959/085 Figure 9)

A sample of the above bromosaquinavir (15 mg) was dissolved in ethanol (200 µl) and stirred vigorously over 10% palladised charcoal in an atmosphere of tritium gas (1 Ci, RC Tritec) for 30 minutes. Hydrogen (5 ml) was then

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introduced and the stirring was continued for a further 30 minutes. The catalyst was removed by filtration, the filtrate evaporated and exchangeable tritium in the residue removed by dissolution in ethanol (2 ml) and re-evaporation (3 times). The product contained approximately 60% of the starting material and was purified chromatographically on a Whatman partisil ODS3 semipreparative column using an aqueous acetonitrile gradient containing 0.1% trifluoroacetic acid and a flow rate of 5 ml/minute. The sample was dissolved in a mixture of ethanol (200 µl) and the initial buffer (38% acetonitrile, 200 µl) and a single injection was applied to the column . The percentage of acetonitrile was increased to 48% over 15 minutes and the product (1.84 mCi, 740 µCi/mg, 500 mCi/mmole) eluted between 8 and 11 minutes. The chemical purity was > 96% and the radiochemical purity > 99%

N-tert-Butyl-decahydro-2-[2-(R)-hydroxy-3(S)-[[N-[3-(4-hydroxyphenyl)-propionyl]-L-asparaginyl]amino]-4-phenylbutyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide (Ro 32-2446)

2-[3(S)-[(L-Asparaginyl)-amino]-2(R)-hydroxy-4-phenylbutyl]-N-tert-butyl-decahydro(4aS,8aS)-isoquinoline-3(S)-carboxamide (Ro 32-0445, Figure 10, ~ 100 mg) was dissolved in dry tetrahydrofuran (1 ml) and a solution of 3-(4-hydroxyphenyl)propionic acid (1, 34.4 mg) in tetrahydrofuran (0.5ml) added. The mixture was stirred at 0°C and treated successively with 1-hydroxybenzotriazole (28.2 mg) in tetrahydrofuran (0.5 ml), N-ethylmorpholine (31 µl, 28.3 mg) and dicyclohexylcarbodiimide (42.2 mg) in more tetrahydrofuran (0.5 ml). The mixture was stirred for one hour at 0°C, and then for three days at room temperature. After filtration, the solvents were removed by evaporation and the residue dissolved in ethyl acetate (5 volume) and re-filtered. The filtrate was washed with saturated aqueous sodium bicarbonate solution (2 x 10 ml) and brine (10 ml). The dried solvent was then evaporated to dryness and the product purified by normal phase HPLC, using the following conditions: column : Waters µ-porasil 250 x 6.25 mm, flow rate : 2 ml/min, eluent : 5% methanol in dichloromethane, detection : UV (280 nm). The partially purified material was combined with the product from the filtration of the crude reaction mixture (after partition between ethyl acetate and aqueous sodium bicarbonate as above) and re-purified by reverse phase HPLC, using the following conditions: column : Whatman Magnum partisil ODS-3 500 x 25 mm, flow rate : 3.4 ml/min, eluent : 50% methanol in 0.02 M aqueous ammonium formate, pH 3.5, detection : UV (254 nm). After evaporation and freeze-drying, the combined yield of the various fractions was 128 mg (73%) and the NMR spectrum was consistent with the proposed structure.

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N-tert.Butyl-decahydro-2-[2-(R)-hydroxy-3(S)-[[N-3-(3-125I-iodo-4-hydroxyphenyl)-propionyl]-L-asparaginyl]amino]-4-phenylbutyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide (Ro 32-1221)

The above compound (19.7 mg) was dissolved in a 1:1 mixture (10 ml) of dimethylformamide and 0.5M phosphate buffer (pH 8.0). A sample of this solution (10 µl) was injected into a solution of sodium 125-iodide (1 mCi in 10 µl dilute aqueous sodium hydroxide, pH ~9), followed by "Chloramine-T" (10 µl of a solution of 53 mg in 10 ml of water). After centrifugation for about one minute, the reaction was stopped by the addition of sodium metabisulphite (0.64 mg in 200 µl of water). After centrifugation for a further 2 minutes the mono-iodo derivative was isolated by reverse phase HPLC, using the following conditions:- column : Whatman partisil ODS-3, 250 x 4.6 mm, flow rate : 1 ml/min, eluent : 63% methanol in 0.02 M aqueous ammonium formate, pH 6.0, detector : Reeve Analytical radiochemical detector with γ cell (10µl). The fraction containing the product (3 ml, 21 % yield) was diluted to 10 ml with 80% methanol in the ammonium formate buffer and stored -20°C. The purity of the material was checked by reverse phase HPLC using the above system, the identity of the labelled derivative being confirmed by comparison with authentic material which had been prepared on a larger scale and whose NMR and mass spectra were consistent with the proposed structure.

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