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THE SYNTHESIS OF LABELLED FORMS OF CIPEMASTAT

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SUMMARY

The development of the collagenase inhibitor, cipemastat (Ro 32-3555), required the synthesis of both carbon-14 labelled and deuteriated material for the measurement of the parent drug and drug-related material in biological fluids. In addition, labelled metabolites were needed for both *in vitro* metabolism and analytical studies. This paper describes the synthesis of labelled forms of cipemastat and its amide (Ro 32-4778) and glucuronide (Ro 32-6414) metabolites.

Key words: Cipemastat, collagenase inhibitor, matrix metallo-proteinase [^{14}C], [^{2}H].

INTRODUCTION

Rheumatoid arthritis is a chronic immuno-inflammatory disease, whose symptoms are associated with inflammation of the synovial membranes (synovitis), which causes pain, tenderness and swelling of the affected joints. In addition, progressive erosion of the cartilage and bone leads to joint destruction and ultimately long-term disability. Currently available therapies for rheumatoid arthritis target the signs and symptoms of the disease but, while they have some impact on joint damage, it is clear that the disease continues to progress in spite of treatment. Cartilage consists of a matrix of glycoproteins supported by a network of collagen in the form of a triple helix. During

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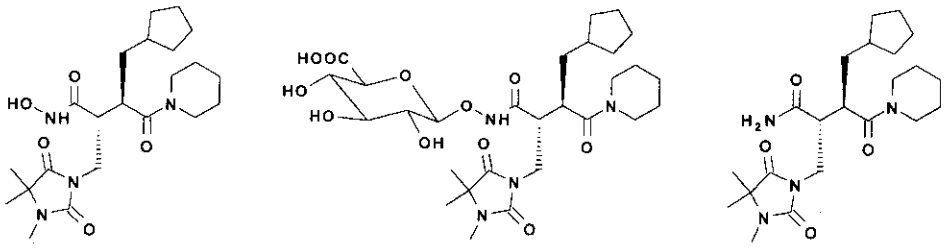
rheumatoid arthritis, increased levels of matrix metallo-proteinases (MMPs), including collagenases, lead to accelerated breakdown of cartilage and, ultimately, destruction of the joint. The key irreversible event in this process is the collagenase-mediated cleavage of the triple helix of collagen at a single peptide bond.

Cipemastat (1-[3-cyclopentyl-2(R)-[1(R)-(hydroxycarbamoyl)-2-(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]propionyl]piperidine, Figure 1) is a potent and selective competitive inhibitor of human collagenases and was specifically designed to inhibit the three human enzymes and thereby prevent the breakdown of cartilage [1]. Its hydroxamic acid function binds tightly to the zinc atom, which is present in the active site of all MMPs, whereas the rest of the molecule confers specificity. The drug has good oral bioavailability [1], [2] and has exhibited activity in models of both rheumatoid arthritis [3] and osteoarthritis [4].



Cipemastat (Ro 32-3555) Ro 32-6414 Ro 32-4778

Figure 1 The structure of cipemastat and its major metabolites

In common with other hydroxamic acids [5], the major metabolites of cipemastat are formed by conjugation and reduction to give a glucuronide (Ro 32-6414) and amide (Ro 32-4778) respectively.

A major requirement of the development programme was an assay to determine plasma and urinary levels of the three compounds. Since they have no suitable UV

chromophore, an HPLC/MS/MS assay was clearly the most appropriate and this necessitated the synthesis of deuteriated compounds with molecular weights several Daltons higher than those of the parent compounds. In addition, carbon-14 labelled cipemastat was needed for standard drug metabolism and pharmacokinetic studies. Several of the final metabolites of cipemastat are derivatives of Ro 32-4778 and it was, therefore, necessary to prepare this compound with a carbon-14 label in order that the relative susceptibility of it and cipemastat to drug-metabolising enzymes could be compared experimentally.

RESULTS AND DISCUSSION

Synthetic strategy

Cipemastat has been prepared by several converging syntheses, e.g. [1], in which the central cyclopentylpropionate moiety is coupled to the piperidine and substituted hydantoin groups (in either order) before addition of hydroxylamine (e.g. Figure 2, Figure 3). The expense of carbon-14 labelled intermediates means that they are ideally incorporated at a late stage of a synthesis and derived from intermediates containing a single carbon atom. These requirements suggested the N-methyl group and the methylene bridge between the hydantoin and the rest of the molecule as likely candidates for the radioactive atom. However, N-methyl groups are often lost by oxidative metabolism and the introduction of the methylene group with labelled formaldehyde was unattractive as the reaction only proceeded well in the presence of excess formalin. Consequently, the relatively long synthesis shown in Figure 2, which introduces the piperidine group before the hydantoin, was developed.

$[^2H_{11}]$ piperidine is commercially available and decadeuteriated cipemastat was readily prepared using the synthetic route shown in Figure 3 where the piperidine

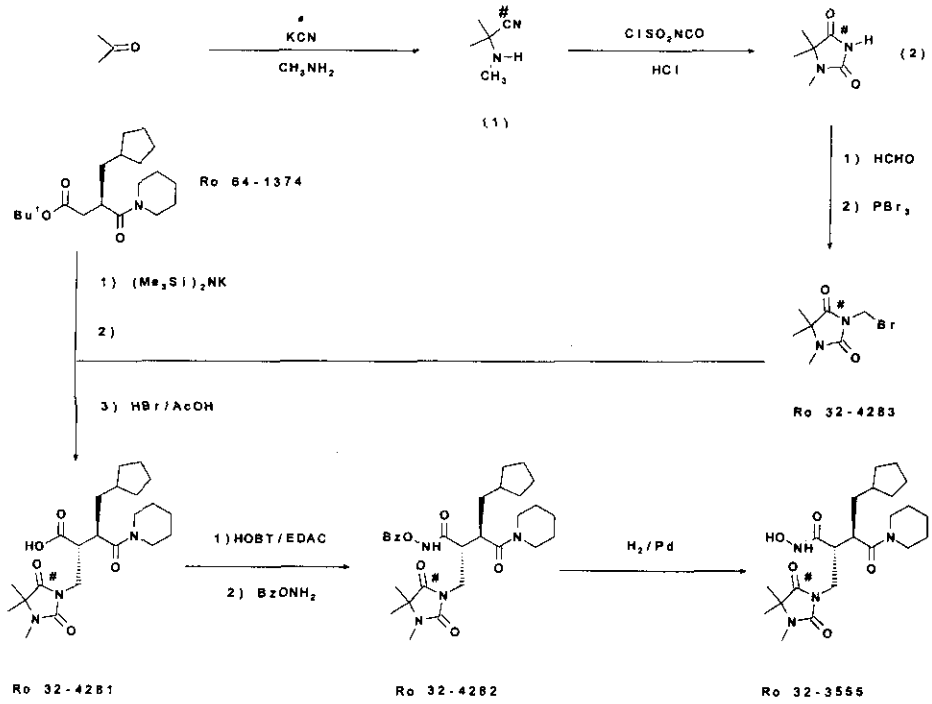


Figure 2 The synthesis of carbon-14 labelled cipemastat

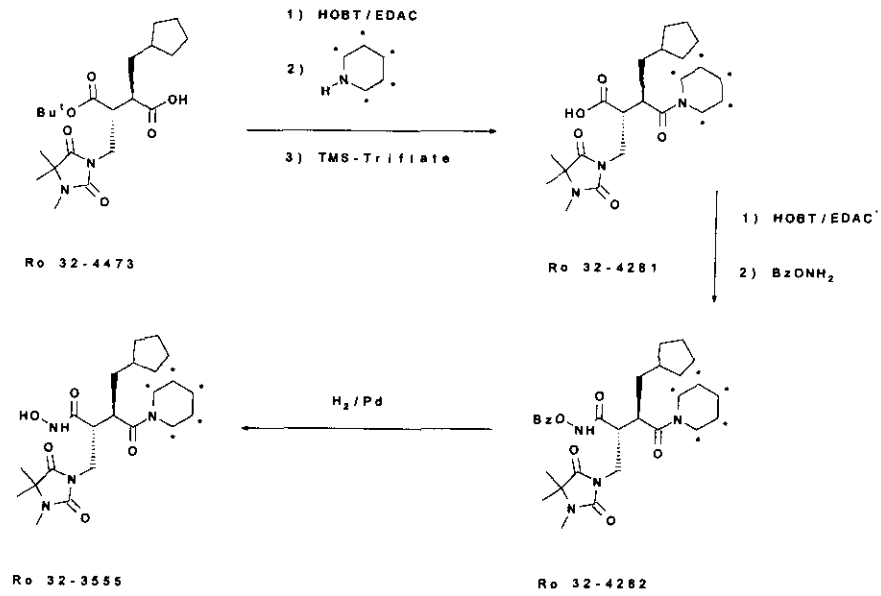


Figure 3 The synthesis of decadeuterio-cipemastat (Ro 32-3555/002)

group is added after the hydantoin. Although the glucuronide of cipemastat can be synthesised, it was found easier to isolate it from the urine of marmosets which had been dosed with cipemastat (or decadeuterio-cipemastat) as this species excretes relatively large amounts of the metabolite into urine.

Synthesis of carbon-14 labelled cipemastat (Ro 32-3555/001)

Labelled potassium cyanide (100 mCi, 55 mCi/mM) was diluted with "cold" material and condensed with acetone and methylamine. The resultant aminonitrile (1, Figure 2) was then converted to the hydantoin (2, Figure 2), in an overall yield of 31%, by reaction with chlorosulphonylisocyanate and hydrochloric acid [6]. The hydantoin was reacted with formaldehyde to give the hydroxymethyl derivative quantitatively. This was separated chromatographically from polymerised formaldehyde and converted to its bromo-derivative (Ro 32-4283) with phosphorus tribromide. The substituted succinate, Ro 64-1374 (Figure 2) was converted to its carbanion with potassium bistrimethylsilylamide, condensed with Ro 32-4283 and deprotected to give the carboxylic acid, Ro 32-4281, in 72% yield. O-Benzylcipemastat (Ro 32-4282) was prepared, *via* an active ester, and purified chromatographically in 86% yield. Since cipemastat is radiochemically unstable, particularly when its specific activity > 10 μCi/mg, it was prepared as required by hydrogenation over palladised charcoal. The yield of the hydrogenation was quantitative and the radiochemical purity of the product > 97%.

Synthesis of decadeuterio-cipemastat (Ro 32-3555/002)

The resolved succinate (Ro 32-4473) was condensed with commercially available [$^2H_{11}$] piperidine *via* an active ester in 89% yield (Figure 3). Elaboration of the

product to Ro 32-4281 and on to cipemastat was uneventful and the overall yield from piperidine was 39%.

Synthesis of decadeuterio-cipemastat-amide (Ro 32-4778/002)

This was prepared similarly from the deuteriated piperidide (Ro 32-4281) by treatment of its hydroxybenzotriazole ester with ammonia in 75% yield.

Synthesis of [^{14}C] labelled cipemastat-amide (Ro 32-4778/001)

No problems were anticipated with the preparation of this compound along the same lines used for radioactive cipemastat and the deuteriated amide. However, several side-products, both radioactive and unlabelled, were formed. In addition, evaporation of the product-containing fractions caused rapid decomposition to a less polar compound. Accordingly, the amide was quickly diluted with an equal weight of unlabelled material to reduce its specific activity, repurified by HPLC and care taken to ensure that evaporations were performed at the minimum temperature and for the minimum time. As soon as a constant dry weight was measured, the material was stored at - 20°C in ethanol-toluene solution. Only a 27% yield was achieved for this step. Various pieces of experimental evidence have led to the conclusion that derivatives of cipemastat can form imides by displacement of the piperidine group (Figure 4). The rate of this reaction appears to be increased by the presence of a

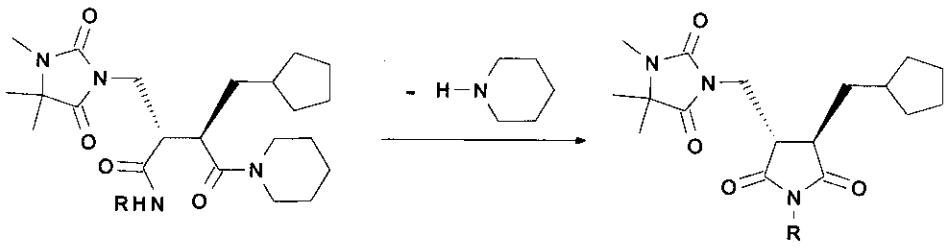


Figure 4 Intra-molecular cyclisation of carbon-14 labelled Ro 32-3555 (R=OH) and Ro 32-4778 (R=H)

carbon-14 group, particularly if the specific activity is relatively high, and is more facile in the case of Ro 32-4778 than Ro 32-3555.

Preparation of cipemastat-glucuronide (Ro 32-6414/001 and Ro 32-6414/002)

Unlabelled cipemastat, or its decadeuterio-derivative, was administered, with a little carbon-14 labelled material to help detection of the metabolites, to female marmosets by oral gavage at 400 mg/kg b.i.d. for 3 days. A total of approximately 4 g of drug was administered to the marmosets and urine was collected from immediately after the first dose until 6 hours after the last one. The recovery of urinary radioactivity corresponded to 34% of the original dose. The glucuronide was isolated by preparative HPLC on a C18 Novapak cartridge using an acetonitrile/aqueous ammonium acetate gradient. Four fractions containing unchanged cipemastat and its major metabolites were pooled according to their retention times. The recovery of drug-related material from the urine was 85% of which 20% was unchanged cipemastat and 55% (770 mg) the glucuronide, whose structure was confirmed by HPLC/mass spectrometry and by NMR.

EXPERIMENTAL

All tetrahydrofuran (THF) 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.

Synthesis of carbon-14 labelled Ro 32-3555/001

1,5,5-Trimethyl-[4-^{14}C]hydantoin [6]

[^{14}C] Labelled potassium cyanide (Amersham International, 100 mCi @ 55 mCi/mM, 1.82 mM) was diluted with unlabelled material (0.142g : 2.18 mM) and reacted with

acetone (0.36g : 6.2 mM) and methylamine hydrochloride (0.34g : 5 mM) in water. The reaction mixture was stirred overnight in a sealed flask and the volatile material collected by vacuum distillation and dried over potassium carbonate. The dry aminonitrile (1, Figure 2) was then vacuum transferred to a liquid nitrogen-cooled flask containing chlorosulphonylisocyanate (0.56g : 4 mM) which was allowed to warm from -40°C to 0°C over 1 hour. After removal of the solvents, the product was heated under reflux in 25% hydrochloric acid for 3 hours. Extraction with dichloromethane gave the [^{14}C] labelled hydantoin (2, Figure 2) in 31% yield. This was diluted with unlabelled material to reduce the specific activity to about 5 mCi/mM.

3-Bromomethyl-1,5,5-trimethyl-[4-^{14}C]hydantoin (Ro 32-4283)

Diluted hydantoin (2, Figure 2) (0.62g : 4.4 mM) was refluxed with 10% formalin (2 ml) overnight after which time no starting material remained. Evaporation and chromatography on silica gel, eluting with 2 : 1 ethyl acetate/hexane, gave a quantitative yield of 3-hydroxymethyl-1,5,5-trimethylhydantoin. This compound (0.50g : 2.9 mM) in molecular sieve-dried dichloromethane (6 ml) was treated with phosphorus tribromide (0.40g : 1.45 mM) at 0°C for 3 hours. After addition to a mixture of ice and water (~ 20 g), extraction with dichloromethane and a bicarbonate wash, the crude product was purified by column chromatography on silica, using 1 : 1 ethyl acetate/hexane, to give 0.574g (84%).

1-[2(R)-[1(R)-Carboxy-2-(3,4,4-trimethyl-2,5-dioxo-1-[5-^{14}C]imidazolidinyl)ethyl]-3-cyclopentylpropionyl] piperidine (Ro 32-4281).

The succinate (Ro 64-1374, Figure 2) (1.59g : 4.92 mM) in anhydrous THF (6 ml) was added to a solution of potassium bis(trimethylsilyl)amide (0.984g : 4.92 mM) in

anhydrous THF at –76°C under nitrogen. After 30 minutes, a solution of the above [^{14}C] bromomethylhydantoin (0.574g : 2.44 mM) in anhydrous THF (5 ml) was added dropwise over 20 minutes. After a further 1.5 hours at –76°C, a 1:1 mixture of brine and 2M hydrochloric acid (10 ml) was added and the mixture allowed to warm to room temperature. The organic layer was removed and the aqueous solution extracted further with ether (2 x 10 ml). The combined organic layers were washed with brine, dried over magnesium sulphate and evaporated to a yellow gum. Column chromatography on silica, eluting with 1:1 ethyl acetate/hexane, gave 0.837g (72%) of the required product. This tertiarybutyl ester (0.835g : 1.75 mM) was stirred in ethyl acetate (7 ml) at 0°C under nitrogen and 45% hydrogen bromide in acetic acid (1.25 ml) added in one portion. After 1.5 hours at 0°C, 1M aqueous sodium hydroxide (6 ml) was added and the solution diluted with water (10 ml) and extracted with ethyl acetate (10 ml). The organic layer was washed with water (2 x 5 ml) and the washes back-extracted with ethyl acetate (2 x 5 ml). The combined ethyl acetate layers were rewashed with water (5 ml) before being dried and evaporated to give 0.77g (slightly over theory) of the acid (Ro 32-4281, Figure 2).

1-[2(R)-[1(R)-(Benzyloxycarbamoyl)-2-(3,4,4-trimethyl-2,5-dioxo-1-[5-^{14}C]imidazolidinyl)ethyl]-3-cyclopentylpropionyl]piperidine (Ro 32-4282).

The above acid (0.77g : ~1.75 mM) was dissolved in dry dichloromethane (16 ml) at 0°C under nitrogen. N-ethylmorpholine (0.45 ml : 3.5 mM), hydroxybenzotriazole (0.32g : 2.1 mM) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.40g 2.1 mM) were added and the mixture stirred at 0°C for 30 minutes. O-Benzylhydroxylamine (0.43g : 3.5 mM) was added and the mixture stirred at 0°C for 15 min and stood at 4°C overnight. After dilution with more dichloromethane

(30 ml), the solution was successively washed with saturated aqueous sodium bicarbonate (10 ml), dilute hydrochloric acid (10 ml) and more saturated bicarbonate solution (10 ml) before being dried and evaporated. The crude product was purified by column chromatography on silica, eluting with ethyl acetate. A total of 0.73g (78%) of the benzylhydroxamate (Ro 32-4282) was obtained.

1-[3-cyclopentyl-2(R)-[1(R)-(hydroxycarbamoyl)-2-(3,4,4-trimethyl-2,5-dioxo-1-[5-^{14}C]imidazolidinyl)ethyl]propionyl]piperidine [5-^{14}C] (Ro 32-3555/001)

Ro 32-4282 (0.19 g : 0.36 mM) in methanol (4 ml) was hydrogenated over 10% palladium on charcoal (~ 25 mg) for 3 hours. The catalyst was removed by filtration and the filtrate evaporated to give Ro 32-3555/001 in quantitative yield. The total radioactivity isolated was 1.91 mCi at 5.6 mCi/mM and the radiochemical purity was >99% by HPLC (3μm Hypersil ODS 12.5 x 0.46 cm, (45-90 %) acetonitrile - 0.05M ammonium acetate gradient over 20 min, flow 1 ml/min, UV @ 219 nm, retention time 3.45 min). The product was stored at -20°C in toluene solution.

Synthesis of deuteriated Ro 32-3555/002

1-[2](R)-[1-(R)-Carboxy-2-(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]-3-cyclopentylpropionyl] [$^{2}H_{10}$]-piperidine (Ro 32-4281)

The resolved succinate (Ro 32-4473, Figure 3) (3.85g : 9.4 mM) in dry dichloromethane (90 ml) was stirred under nitrogen at 0°C and treated with N-ethylmorpholine (2.4 ml : 19 mM) and 1-hydroxybenzotriazole hydrate (1.45 g : 10.7 mM) followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.0g : 10.5 mM). The mixture was stirred at 0°C for 1 hour and $^{2}H_{11}$ piperidine (Cambridge Isotopes, 1.0 g : 10.5 mM) added. The mixture was stirred at

0°C for 2 hours and then at room temperature overnight. After being washed with dilute sulphuric acid (30 ml) and aqueous sodium bicarbonate (2 x 30 ml), the solution was dried and evaporated to give the $^{2}H_{10}$-piperidide (4.45g , 89%).

The tertiary butyl group was removed by heating a solution of the ester in dry dimethoxyethane (30 ml) with triethylamine (3 ml : 21.8 mM) and trimethylsilyl trifluoromethanesulphonate (3.5 ml : 4.04 g: 18.2 mM) under reflux for 20 minutes. The cooled reaction mixture was partitioned between ether (30 ml) and water (20 ml) and the aqueous layer extracted with more ether (3 x 40 ml). The combined ether layers were extracted with saturated aqueous sodium bicarbonate (3 x 50 ml). This aqueous solution was acidified with 2N sulphuric acid and extracted with ether (3 x 50 ml). The ether extracts were dried and evaporated to give 3.11g of solid (79%).

1-[3-cyclopentyl-2(R)-[1(R)-(hydroxycarbamoyl)-2-(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]propionyl][$^{2}H_{10}$]piperidine (Ro 32-3555/002)

Decadeuterio-Ro 32-4281 was converted through to decadeuterio-cipemastat as before to give (1.81g, 39% from piperidine).

Synthesis of deuteriated Ro 32-4778/002

1-[2](R)-[1-(R)-Carbamoyl-2-(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)ethyl]-3-cyclopentylpropionyl] [$^{2}H_{10}$] piperidine (Ro 32-4778/002)

The carboxylic acid (Ro 32-4281/000, 3.808g : 10.2 mM, Figure 3) in dichloromethane (35 ml) was cooled in an ice bath before the addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.15g : 11.2 mM) and 1-hydroxybenzotriazole hydrate (1.51g : 11.2 mM). The mixture was stirred at 0°C for 1 hour by which time TLC (2:1 ethyl acetate : hexane on silica) showed no

carboxylic acid remaining. Ammonia solution (0.880, 4.0 ml : 77 mM) was then added and the mixture stirred vigorously for three hours. The reaction was stood at 4°C overnight before being extracted with dichloromethane (20 ml). The organic layer was washed with aqueous sodium bicarbonate and brine, dried and evaporated to leave 3.55 g (81%) of a gum. Column chromatography on silica, eluting with 10% methanol in dichloromethane, gave 3.30g (75%) of a white solid which was > 96% pure by HPLC (5 μm C18 15 x 0.46 cm Hypersil column, 25% acetonitrile/0.05 M triethylamine phosphate, pH 2.5). Mass spectrometry gave almost a single ion at m/e = 431 corresponding to the decadeuterio-amide.

Synthesis of carbon-14 labelled Ro 32-4778/002

1-[2](R)-[1-(R)-Carbamoyl-2-(3,4,4-trimethyl-2,5-dioxo-1-[5-^{14}C]imidazolidinyl)ethyl]-3-cyclopentylpropionyl]piperidine (Ro 32-4778/002)

The [^{14}C] labelled carboxylic acid (Ro 32-4281) (100 mg at ~10μCi/mg : ~0.25 mM) in dichloromethane (1.0 ml) was cooled in ice/water and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (54 mg : 0.28 mM) added followed by hydroxybenzotriazole hydrate (38 mg : 0.29 mM). After stirring for 30 min, aqueous saturated ammonia (0.1 ml) was added. The cooling bath was removed and the mixture stirred overnight before being partitioned between water (1 ml) and dichloromethane (2 ml). The aqueous solution was further extracted with dichloromethane (2 x 1 ml) and the combined organic layers were washed with saturated sodium bicarbonate and brine (2 ml of each) before being dried and evaporated to a light brown oil (104 mg, > 95% crude yield). This was purified by reverse phase preparative HPLC on a Waters μBondapack C18 RCM cartridge (10 x

2.5 cm) eluting with an acetonitrile / 0.05M triethylammonium phosphate (pH 2.5) buffer gradient (10 min at 10% acetonitrile then linear gradient from 10% to 90% over the next 20 minutes) with a flow rate of 5 ml/min. The radioactive peak eluting at 8 min was collected. Concentration to ~2/3 volume and extraction with ethyl acetate gave, after evaporation a brown oil. Analytical HPLC showed a large impurity. The material was diluted with 100 mg of unlabelled product and rechromatographed using the same conditions. Analytical HPLC now showed just a close running impurity (~ 5%) and so the material was purified a third time using a Phenomenex Columbus semi-preparative C18 column (25 x 1 cm) eluting with a linear 30-70% acetonitrile / 0.05M triethylammonium phosphate buffer (pH 2.5) gradient over 20 min. The product-containing fractions were combined and concentrated to approximately half volume and extracted with ethyl acetate (4 x 5 ml). The extracts were dried over anhydrous potassium carbonate and evaporated to dryness at room temperature *in vacuo* to leave 28 mg of solid (27%) which was immediately dissolved in 10% ethanol in toluene for storage at – 20°C. The radiochemical purity was > 99% by analytical HPLC and the specific activity was measured as 9.9 μCi/mg.

Preparation of unlabelled and deuteriated Ro 32-6414

1-O-[4-cyclopentyl-2(R)-[3,4,4-trimethyl-1imidazolidinylmethyl]3-(R)-(piperidinocarboxybutyramido]-ß-D-glucopyranouronic acid (Ro 32-6414/000)

Unlabelled cipemastat, or its decadeuterio derivative, (~ 5 g) was suspended with carbon-14 labelled material (~ 300 μCi) in 0.2% aqueous carboxymethylcellulose and 0.1% Tween 80 in pH 4 acetate buffer at a nominal concentration of 50 mg/g. Four

female marmosets with a bodyweight range of 330 – 500 g were dosed by oral gavage at 400 mg/kg b.i.d. for 3 days and given a final dose on Day 4. A total of approximately 4 g of drug was administered to the marmosets and urine was collected from immediately after the first dose until 6 hours after the last one. The recovery of urinary radioactivity corresponded to 34% of the original dose and the urinary samples from the different animals were pooled by collection time (0-6 h, 6-24 h, 24-48 h, 48-72 h).

Each pooled urinary sample was separately applied to a C_8 MegaBond-Elut™ cartridge that had been conditioned by washing with acetonitrile (50 ml) and water (2 x 50 ml). The urine was allowed to drip slowly through the cartridges under gravity to maximise retention of drug-related material. The cartridges were then washed with water (3 x 20 ml) and the radiolabelled material eluted with 80% acetonitrile in 0.5 M ammonium acetate buffer (pH 3.5 ; 1 x 20 ml, 1 x 10 ml) in >90% yield. Almost all the eluent was evaporated and the residues dissolved in 10% acetonitrile in 0.01M ammonium acetate buffer (pH 3.5). The drug-related compounds were separated using preparative HPLC on a Waters C_{18} Novapak™ cartridge (250 x 10 mm) in a Radial Compression Module with a C18 guard column. The purified urine was applied using a 2 ml Rheodyne loop and eluted at 7.5 ml/min with a linear acetonitrile / 0.01M ammonium acetate buffer (pH 3.5) gradient (10% - 30% acetonitrile over 40 min; 30% acetonitrile for 5 min; 30% - 10% acetonitrile over 15 min). The peaks were detected by UV at 235 nm (Kratos Spectroflow 773) and radiochemically (Reeve 9701 with heterogeneous cell at 128 cps).

More than 85% of the radioactive material was collected in four fractions. The second fraction was by far the largest, accounting for 55% of the urinary radioactivity. The

acetonitrile from this fraction was evaporated off and the remaining solution freeze-dried to give 770 mg. NMR and mass spectrometry confirmed this material as the glucuronide. Later fractions were identified as the glucuronide of the carboxylic acid, formed by formal hydrolysis of Ro 32-3555, and the parent compound.

CONCLUSIONS

This paper describes the synthesis of labelled forms of cipemastat and its two major metabolites, Ro 32-4778 and Ro 32-6414 using related, convergent syntheses that introduce the label relatively late. The carbon-14 labelled compounds were satisfactorily prepared despite problems of radiochemical instability, particularly with Ro 32-4778. The three decadeuterio-analogues, which were needed for the establishment of HPLC/MS/MS assays for pharmacokinetic studies, were efficiently synthesised from piperidine. Both deuteriated and non-labelled Ro 32-6414 (the glucuronide conjugate) were isolated from marmoset urine after the animals had been dosed with the corresponding form of cipemastat.

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