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^{11}C-Radiosynthesis and preliminary human evaluation of the disposition of the ACE inhibitor [^{11}C]zofenoprilat

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Abstract—(4*S*)-1-[(*S*)-3-Mercapto-2-methylpropanoyl]-4-phenylthio-L-proline (Zofenoprilat, **2**), the active metabolite of the potent ACE inhibitor Zofenopril Calcium (**1**), was labelled with carbon-11 ($t_{1/2}$ = 20.4 min) to evaluate its pharmacokinetics behaviour in human body using Positron Emission Tomography (PET). [^{11}C]2 labelling procedures were based on the use of immobilized Grignard reagent and the acylation of (*S*)-4-phenylthio-L-proline methyl ester (**5**) with ^{11}C-labelled methacryloyl chloride, followed by a Michael addition with thiobenzoic acid. The radiochemical yield was 5–10% (EOB, decay corrected) and specific radioactivity ranged from 0.5 to 1.5 Ci/μmol (18.5–55.5 GBq/μmol). Preliminary in vivo human evaluation of [^{11}C]**2** showed that the drug accumulates in organs which express high levels of ACE, like lungs and kidneys, and in organs involved in drug metabolism such as the liver and gall bladder. Results of the distribution of [^{11}C]**2** showed a measurable concentration of the drug in the target tissues such as the kidney and to a minor extent, the heart, where it can afford organ protection.

1. Introduction

Positron Emission Tomography (PET) enables the in vivo study of different pharmacological variables in human subjects using methods originally developed for pre-clinical studies in rodents.[1] The radiolabelling of drugs with positron emitters allows the in vivo measurement of drug concentration in different areas of the human body and the in vivo characterisation of a drug's pharmacokinetic behaviour in the target organ. Limits of this strategy are the short half-lives of positron emitting isotopes that ranges from 20.4 min for carbon-11 to 109.8 min for fluorine-18.[2] Thus, the synthetic procedures must be selected in a way that introduction of the label occurs at the final steps of the procedure. After the discovery of Captopril, the prototype of orally active angiotensin converting enzyme (ACE) inhibitors, several new ACE ligands were developed and introduced into medical practice. These agents (Captopril, Analapril, Fosinopril, etc.) differ in their chemical structure, functional groups (presence of sulfhydryl, carboxyl or phosphinyl), active moiety, pharmacological potency and pharmacokinetics. These characteristics differentiate ACE inhibitors from each other and influence their ability to inhibit the enzyme in various organs.[3] Among the various ACE agents, the $^{18}F^-$ radiolabelled fluoro analogue of captopril, 4-[^{18}F]fluoro-1-(3-mercapto-2-methyl-1-oxopropyl)-L-proline (4-*cis*-[^{18}F]fluorocaptopril) has been evaluated as a potential radioligand for PET and was indicated as a new radiopharmaceutical for the in vivo ACE measurement.[4]

(4*S*)-1-[(2*S*)-3-Benzoylthio-2-methyl-1-oxopropyl]-4-(phenylthio)-L-proline (Zofenopril, **1**) (EC_{50} = 6–80 nM) is an ACE inhibitor that is characterised by an high cardio-protective activity supposed to be due to a potent and selective inhibition of myocardial ACE as well as to the free radical scavenging properties of its sulfhydryl group.[5–10] The cardio-protective activity of Zofenopril

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is of relevance for its use in the prevention and therapy of cardiovascular diseases.[11]

To evaluate whether the cardio-protective effect could be linked to a preferential accumulation of the drug in myocardial tissue, we have developed a strategy for the in vivo PET measurement of myocardial uptake of Zofenopril in humans. Zofenopril is a pro-drug that is deesterified at the thioester group to give the active product Zofenoprilat, (4*S*)-1-[(*S*)-3-mercapto-2-methylpropanoyl]-4-phenylthio-L-proline (**2**) (EC_{50} = 1–8 nM) (Fig. 1).

To avoid confusing effects resulting from the radiolabelled circulating pro-drug, we decided to label the active compound Zofenoprilat. Finally, to test the hypothesis of myocardial targeting, the kinetics of myocardial distribution of [^{11}C]zofenoprilat was investigated in a pilot study carried out on a patient with moderate hypertension.

To this aim a new synthetic approach of Zofenoprilat was designed. It was based on the acylation of (*S*)-4-phenylthio-L-proline methyl ester (**5**) with ^{11}C-labelled methacryloyl chloride followed by a Michael addition with thiobenzoic acid. A key feature of the radiochemical synthesis was the use of an immobilized Grignard reagent to obtain ^{11}C-labelled acyl chloride (as published by McCarron et al.[12]) employing a partially modified Nuclear Interface PET Tracer Synthesizer.

2. Results and discussion

2.1. Chemistry

The short-lived positron emitting ^{11}C radionuclide requires that the labelling be introduced in one of the last steps of the synthesis. Hence, the current process (to be published elsewhere) to obtain the precursor Zofenopril **1** was modified. As shown in Scheme 1, the retrosynthetic pathway implies the introduction of the ^{11}C label in a carbonyl group.

The synthesis (Scheme 2) involves the reaction of commercially available isopropenylmagnesium bromide (**3**) with CO_2 to give magnesium bromide methacrylate (**4**). This compound was joined to the proline derivative **5** to give the methacrylamide **6** which can alternatively be prepared starting from methacrylic acid (see Experimental). After purification by flash chromatography (*n*-hexane/EtOAc 6:4), methacrylamide **6** was reacted with thiobenzoic acid in the presence of *N*,*N*-diisopropylethylamine (DIPEA) to give a mixture of the two diastereomers **7** as products of the Michael addition. These isomers were separated by flash chromatography (*n*-hexane/AcOEt 65:35). On the basis of ^{1}H NMR the diastereomer with higher R_f was Zofenopril methyl ester [(*S*,*S*,*S*)**7**]. Zofenoprilat (**2**) and its (*R*,*R*,*S*) diastereomer were finally obtained by hydrolysis with NaOH in MeOH/H_2O. The whole process was then repeated 'one-pot' (see Experimental) without isolation and purification of the intermediates or separation of the two diastereomers **7** in order to reduce the time of the synthesis. The results (<40 min required for the overall process) were considered satisfactory for transferring to the radiosynthesis.

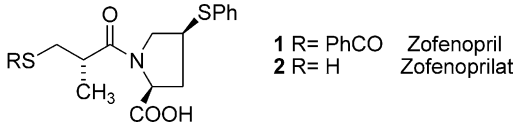
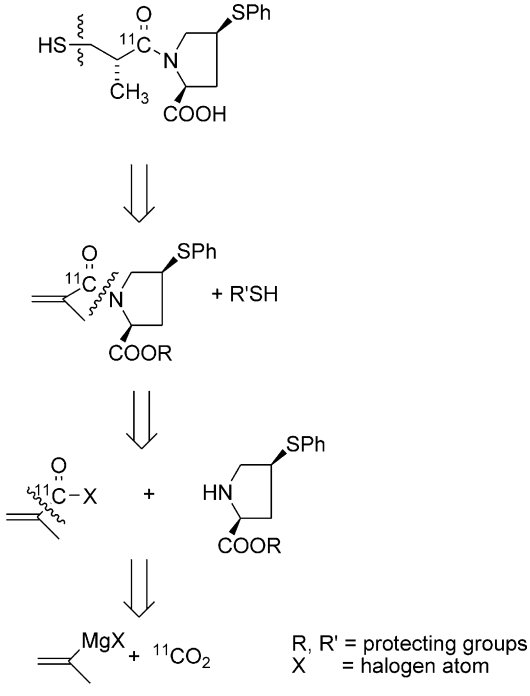


Figure 1. Zofenoprilat and its precursor Zofenopril.

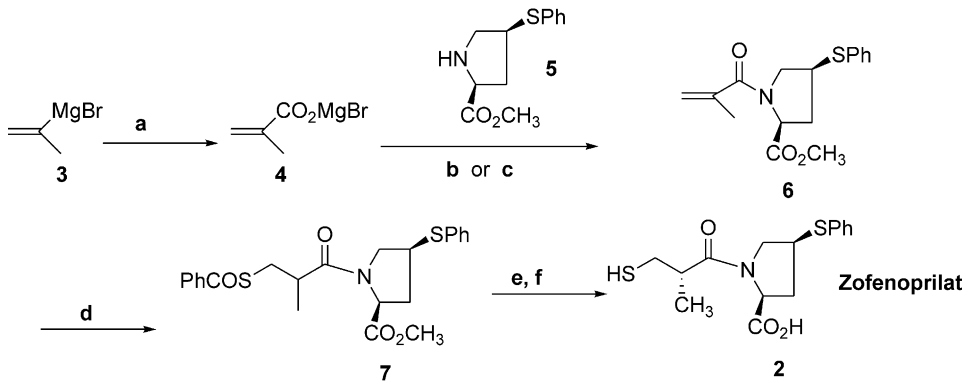
2.2. Radiochemistry

The one-pot procedure developed for the preparation of cold Zofenoprilat was adapted for the synthesis of [^{11}C]zofenoprilat ([^{11}C]**2**) by converting [^{11}C]**4** to radiosynthon **8** to provide the [^{11}C]**6** intermediate. The ^{11}C-carboxylation (Scheme 3) procedure described by McCarron[12] for the radiosynthesis of the [*carbonyl*-^{11}C]cyclohexanecarbonyl chloride, using a modified ^{11}C-methylation radiosynthesis module, was adopted.[13] The described strategy consists of the deposition of a thin film of the Grignard reagent on the inner surface of narrow bore tubing. [^{11}C]Carbon dioxide is then passed through the tube in a stream of inert gas. The reaction to give [^{11}C]**4** occurs at the gas–liquid interface. Formation and recovery of the [^{11}C]acyl chloride **8** is achieved by passing thionyl chloride through the tube, which is directly connected to the reaction vial containing the appropriate precursor **5** for the subsequent [^{11}C]carboxylation reaction.[12] THF was found to be the solvent of choice for [^{11}C]**6** radiosynthesis, provoding a 55% EOS (end of synthesis) radiochemical yield.

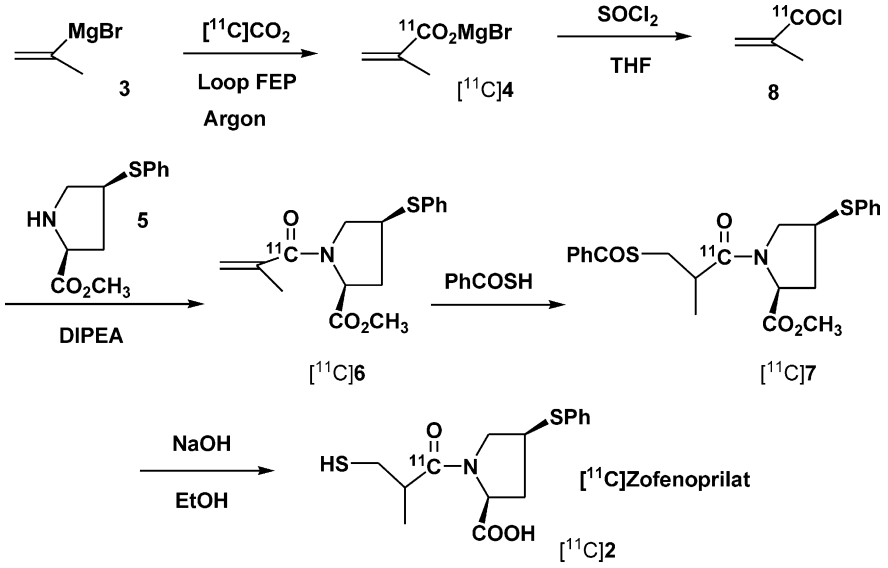
The Michael adduct [^{11}C]**7** was synthesized by addition of thiobenzoic acid (150 μL) to the methacrylamide



Scheme 1. Retrosynthetic analysis of Zofenoprilat.



Scheme 2. Synthesis of Zofenoprilat via Michael addition. Reagents and conditions: (a) CO_2, THF, −10 °C to rt; (b) EDAC·HCl, DIPEA, HOBt, THF, rt; (c) HATU, DIPEA, DMF 55 °C; (d) PhCOSH, DIPEA, 60–95 °C; (e) separation of diastereomers by flash chromatography (*n*-hexane/AcOEt 65:35); (f) 1 N NaOH, H_2O, MeOH or 1,4-dioxane.



Scheme 3. Radiosynthesis of [^{11}C]Zofenoprilat.

[^{11}C]**6** with a radiochemical yield of 65%. Also in this part of the radiosynthesis a mixture of two labelled diastereomers was obtained (as described in the Experimental). An HPLC pre-purification step of the [^{11}C]**7** adduct was performed, thus allowing elimination of the large excess of thiobenzoic acid. This technical solution and subsequent solid phase extraction (SPE) for recovery of the pure Michael adduct [^{11}C]**7** required manual performing of the hydrolysis and final radiopharmaceutical formulation for intravenous administration.

The overall radiochemical yield (EOB, decay corrected) of the [^{11}C]**2** was 5–10%. The specific radioactivity ranged from 0.5 to 1.5 Ci/μmol (18.5–55.5 GBq/μmol), (EOS) with an average time of radiosynthesis of 60 min, including radiopharmaceutical formulation. In a typical experiment starting from 400 to 600 mCi (14.8–22.2 GBq) of [^{11}C]CO_2, 5–10 mCi (185–270 MBq) of the formulated [^{11}C]Zofenoprilat was obtained with 100% radiochemical purity.

[^{11}C]**2** and intermediate Michael adduct [^{11}C]**7** identities were confirmed by co-injection of their non-radioactive authentic standards on reverse-phase HPLC and by the mass spectrometric analyses of the products of their carrier-added radiosyntheses (see Experimental).

2.3. PET study

A rapid and transient increase of radioactivity was observed immediately post-injection in the myocardial wall and in the ventricular cavity (Fig. 2). In these regions maximal radioactivity uptake was observed approximately 1 min after injection. At this time, radioactivity concentration in the myocardial tissue (MT) and ventricular cavity (VC) was equal to 0.008% I.D./mL (0.37 pmol/mL) and 0.014% I.D./mL (0.56 pmol/mL), respectively. Time courses of blood radioactivity concentrations whether measured by PET or direct counting of blood samples gave similar results (Fig. 3) indicating the reliability of PET measurements. Myocardial radioactivity concentration remained below that measured in the LV for the entire period of observation. At the early stages, it displayed a kinetic behaviour similar to that in blood suggesting that, immediately after injection, radioactivity uptake in

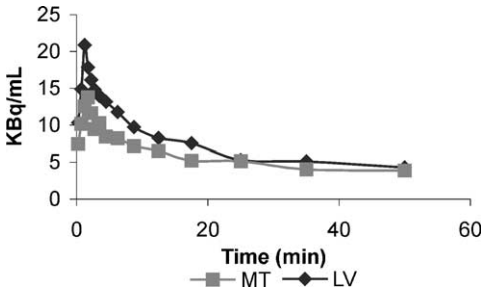


Figure 2. Time–activity curve of [^{11}C]**2** uptake, expressed in absolute radioactivity concentration units, in the heart tissue (MT) and left ventricle cavity (LV) of a patient with moderate hypertension.

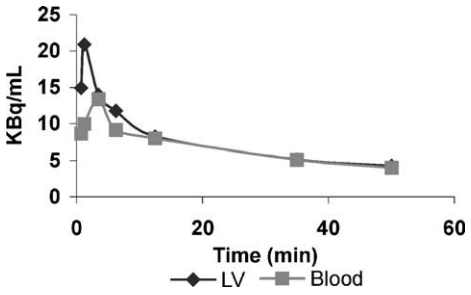


Figure 3. Time–activity curve of [^{11}C]**2** radioactivity uptake, expressed in absolute radioactivity concentration units, in the blood of a patient with moderate hypertension. Blood concentration was measured using both PET (LV) or direct sampling of serial blood aliquots (Blood).

myocardium depends on tissue perfusion or on the spill-over effects of circulating radioactivity in the LV. However, at a later times we observed a progressive increase in myocardial tissue to blood radioactivity concentration ratio (0.9 at 50 min after injection; see Fig. 4) suggesting a slow cardiac accumulation.

A rapid rise in radioactivity concentration was observed in lungs and kidneys. In these organs, radioactivity concentration peaked at 2–3 min after injection and remained fairly stable thereafter (Fig. 5). At the time of maximum uptake, radioactivity concentration in lungs and kidneys was 0.0072 and 0.0073% I.D./mL, respectively. In the liver and gall bladder a progressive rise in radioactivity concentration was also observed, more markedly in the gall bladder.

Radioactivity distribution measured at 50 min after injection is shown in Figure 6. The rank order of radioactivity uptake was: gall bladder > > liver > kidney = lung > blood = myocardium.

Quantitative organ distribution substantially overlaps that previously observed in rats with oral administration of [^{14}C]Zofenopril.[14] In this study, 30 min after drug administration, radioactivity concentration in all tissue except the kidney, small intestine and urinary bladder was lower than that found in plasma while after 24 h the tissue to blood ratios were markedly increased in excretory and cardiovascular tissues. Similarly, in our study at 50 min after injection, the radioactivity concentration in most of the organs sampled was higher or equal to blood concentration. In the two studies different chemical forms were administered ([^{14}C]zofenopril in rat

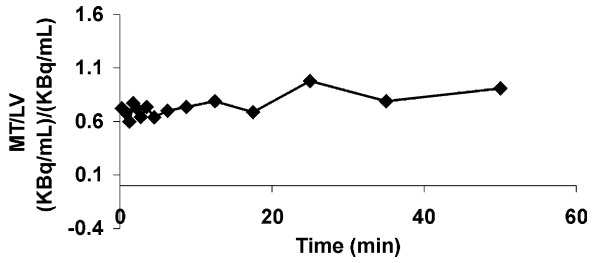


Figure 4. Time–activity curve of heart tissue (MT) to left ventricle cavity (LV) radioactivity concentration ratio measured in a patient with moderate hypertension after the iv injection of [^{11}C]**2**.

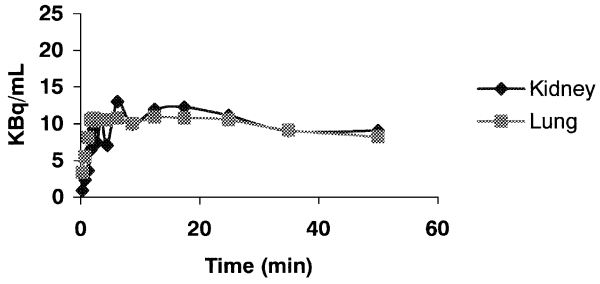


Figure 5. Time–activity curve of [^{11}C]**2** radioactivity uptake expressed in absolute radioactivity concentration units, in lungs and kidneys of a patient with moderate hypertension.

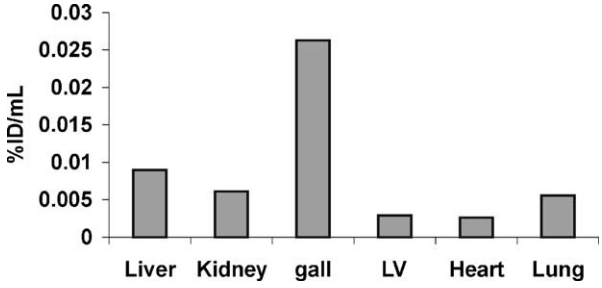


Figure 6. Uptake of [^{11}C]**2** expressed as %I.D./mL, measured at approximately 60 min in different organs of a patient with moderate hypertension.

study versus [^{11}C]zofenoprilat in the human one) and they differed also for the route of administration used (oral versus intravenous). In spite of that, it should be noted that zofenoprilat is the main and the only active metabolite of Zofenopril.

Pre-clinical data showed that Zofenopril was able to accumulate in the heart and vascular tissue[14] and exert a long-lasting local ACE inhibitory activity.[15] Local activation of the renin–angiotensin system and the formation of angiotensin II are reported as risk factors in the development of cardiac hypertrophy and heart failure, myocardial infarction, atherosclerosis and so on[16,17] and therefore the prolonged local inhibition of cardiac ACE is thought beneficial for the prevention of these cardiovascular events. The amount of radioactivity observed in MT during this study and the low concentration of Zofenoprilat (3 nM) necessary to inhibit cardiac ACE[16] suggest that, at pharmacological doses, local enzyme inhibition may be effective.[11]

Likewise, the high radioactivity concentration found in the kidney (peak = 0.0073% I.D./mL) and the ability of Zofenopril to inhibit local ACE in the 1–3 nM range[15] may be the basis for the organ-protection afforded by this drug.

Extrapolating these data in a person (where the common therapeutic antihypertensive dose of 30 mg o.d. produced a C_{max} of 341 ng/mL[18] corresponding to a concentration of 1 μM) we can put forward the hypothesis that in the target tissues such as heart and kidney the concentration reached by the drug is able to block the local production of the ACE.

In conclusion, the results of this study, which for the first time demonstrate the in vivo measurements of [^{11}C]Zofenoprilat in the human body, confirm those previously reported in rodents indicating that the drug accumulates in target organs where ACE is more expressed.[16,17] In fact, in spite of the obvious limitation of a pilot study conducted on one single patient, PET data indicate that also in the human body the drug accumulates in organs that express high levels of ACE, like lungs, kidneys and to a minor extent in myocardium.

Our PET biodistribution study was performed on a diastereomeric mixture of radiolabelled Zofenoprilat and not on the (*S,S,S*) isomer which is the main metabolite of the form currently used clinically. In vitro studies on the inhibitory activity of (*R,S,S*)- and (*S,S,S*)-Zofenoprilat on ACE indicate that (*S,S,S*)-Zofenoprilat is 156 times more potent than (*R,S,S*)-Zofenoprilat [IC_{50} values of 10.9 and 1700 nM, respectively (Lusochimica, internal report)]. Thus, on the basis of the above results, we can reasonably hypothesise that the administration of a mixture of radiolabelled epimers instead of [^{11}C](*S,S,S*)-Zofenoprilat has reduced the levels of specific uptake and signal to noise ratios in organs expressing ACE.

However, due to the short half life of the radioisotopes, PET-based pharmacokinetic studies do not allow monitoring of drug distribution for more than 1 h after injection and it is not possible to evaluate in depth the further distribution of the drug.

3. Experimental

3.1. Chemistry

Reagents and solvents were purchased from common commercial suppliers and used without purification. Isopropenylmagnesium bromide (0.5 M in THF) was purchased from Aldrich (Milano, Italy). (*S*)-4-Phenylthio-L-proline was prepared by Lusochimica. Unlabelled reference substances were prepared by Lusochimica and used for comparison in all the HPLC runs in the analyses of the ^{11}C-labelled compounds. Authentic sodium Zofenoprilat was obtained from Berlin Chemie (Berlin, Germany). Identities of all the unlabelled synthesised materials were confirmed using ^{1}H NMR, MS and elemental analysis. ^{1}H NMR spectra were recorded at 200 MHz on a Bruker AC-200 or a Bruker CXP-400 spectrometer in indicated solvent; chemical shifts are reported as δ (ppm) relative to TMS as external standard. The mass spectra were recorded in positive ion mode with a VG 7070 EQ-HF mass spectrometer. The infrared spectra were recorded on a Perkin-Elmer FT-IR 1600 spectrometer. Elemental analyses were performed on Fison Instrument EA-1108. TLC was performed on silica-coated glass plates (Merck, Silicagel 60 F_{254}) in the indicated solvent mixture, using UV light as detector unless otherwise noted. Flash chromatography was performed with silica gel 0.040–0.063 μm using the indicated solvent mixture. Analytical HPLC was performed with a Merck-Hitachi LaChrom system equipped with a C-18 column (Waters μBondapack, 10 μm, 300×3.9 mm); flow rate 1 mL/min, λ 238 nm; mobile phase MeOH/0.05 M KH_2PO_4, pH 3 70:30 (v:v); temperature 31 °C. Semi-preparative HPLC was performed with a Waters 600 E system equipped with a Waters 486 UV detector, a Shimatzu C-R6A Chromatopac integrator and a C-18 column (Waters μBondapack, 10 μm, 300×19 mm); flow rate 17 mL/min; λ 238 nm; mobile phase MeOH/0.05 M KH_2PO_4, pH 3 50:50 (v:v); temperature 25 °C.

[^{11}C]Carbon dioxide was produced by the ^{14}N(p,α)^{11}C reaction on a CTI-Siemens RDS-112 cyclotron, using a 11.5 MeV proton beam at currents of 10–30 μA, and trapped in a hollow stainless steel loop cooled with liquid nitrogen. Radiochemical syntheses were performed on modified fully automated synthesis module (PET Tracer Synthesizer, Nuclear Interface Datentechnik GmbH, Münster, Germany). HPLC of labelled compounds was performed with a Waters Millennium system equipped with UV detector set at 238 nm, a flow radioactivity detector and a reversed-phase analytical HPLC column (Shandon Hypersil BDS C-18, 5 μm, 250×4.6 mm). pH of solutions was measured with a Shott-Geräte pH-meter.

Identity of the final ^{11}C-labelled compounds was assessed by MS. Dried samples were reconstituted with 50 μL methanol and aliquots were analysed by APCI/MS. Samples were introduced by a Rheodyne injector with a loop of 20 μL using water/methanol 1:1 (v:v) as delivering solvent (flow rate, 300 μL/min). The APCI spectra were recorded with a Finnigan MAT95. LC-APCI/MS was performed with a Beckman Ultrasphere ODS column (150×46 mm, 5 μm) using methanol/water 1:1 (v:v) containing 0.1% acetic acid at flow rate of 1 mL/min generated with a Jasco 880-PU HPLC pump (Jasco Spectroscopic Co. LTD, Tokyo, Japan). The eluent was directly introduced into the APCI source of a Finnigan MAT95 double focusing mass spectrometer. The corona discharge was set at 2 μA, the vaporizer at 350 °C and the heated capillary at 200 °C. Mass spectrometry conditions were as follows: capillary voltage 3.1 KV and 38 V, tube lens 60 V, skimmer voltage 4.2 V and octapole voltage 1 V. Spectra were recorded by scanning from *m/z* 100 to *m/z* 800 at 3 s/dec. Skimmer-CID spectra were recorded increasing tube lens and the heated capillary voltage, respectively.

3.1.1. Magnesium bromide methacrylate (4). A 250-mL four-neck round-bottom flask equipped with magnetic

stirrer and thermometer was inertized with N_2 stream and charged with 40 mL (20 mmol) of isopropenylmagnesium bromide (0.5 M solution in THF) (3). An excess of $CO_{2(g)}$ was bubbled in the solution which cooled to −10 °C over 5 min. An exothermic effect was observed which increased the temperature to rt. The resulting solution of raw magnesium bromide methacrylate (20 mmol) (4) was used without further purification. For analytical purpose an amount was treated with 0.1 N HCl, extracted with CH_2Cl_2 and the organic solution was evaporated. The ^{1}H NMR spectrum of the residue agreed with literature data[19] HPLC: t_R 3.2 min. ^{1}H NMR ($CDCl_3$): δ 6.25 (dq, J = 1.6, 1.0 Hz, 1H, *H*CH=); 5.68 (dq, J = 1.6, 1.6 Hz, 1H, HC*H*=); 1.95 (dq, J = 1.6, 1.0 Hz, 3H, CH_3).

3.1.2. (*S*)-4-Phenylthio-L-proline methyl ester (5). 15.0 g (67 mmol) of (*S*)-4-phenylthio-L-proline was dissolved in 150 mL of methanol. 10 mL (79 mmol) of trimethylsilyl chloride was added and the mixture was heated to reflux. The reaction was monitored by TLC (CH_2Cl_2/MeOH/TEA 88:10:2). After 10 h, no starting material was detected. The solvent was evaporated under vacuum. The resulting residue was dissolved in 90 mL of CH_2Cl_2. The mixture was neutralised by adding 70 mL of 1 N $NaHCO_3$. The aqueous phase was separated and extracted with CH_2Cl_2 (2×80 mL). The combined organic solutions were washed with brine (70 mL), dried over Na_2SO_4 and filtered. The solvent was removed under vacuum to give 15.4 g (97%) of **5** as yellow oil. TLC (EtOAc/MeOH 8:2): R_f 0.47. HPLC: t_R 3.4 min. ^{1}H NMR ($CDCl_3$): δ 7.4–7.2 (m, 5H, aromatic H); 3.9–3.7 (m, 2H, C*H*CO_2CH_3, C*H*SPh); 3.75 (s, 3H, CH_3); 3.22 (dd, J = 11.6, 6.1 Hz, 1H, *H*CHN); 3.05 (dd, J = 11.6,5.1 Hz, 1H, HC*H*N); 2.56 (ddd, J = 13.7, 8.8, 7.3 Hz, 1H, *H*CHCHCO_2CH_3); 2.24 (bs, 1H, NH); 1.93 (ddd, J = 13.7, 5.9, 5.9 Hz, 1H, HC*H*CHCO_2CH_3). MS (EI) m/z 237 $[M]^+$. Preparation of the hydrobromide salt of **5** was reported in literature[20] but no analytical data were reported.

3.1.3. (4*S*)-1-(2-Methylpropenoyl)-4-phenylthio-L-proline methyl ester (6). 7.5 g (32 mmol) of (*S*)-4-phenylthio-L-proline methyl ester (**5**) was dissolved in 75 mL of THF. To the resulting solution under stirring, 5.15 g (33 mmol) of 1-hydroxybenzotriazole, 17 mL (95 mmol) DIPEA, 2.90 mL (33 mmol) methacrylic acid and 8.1 g (41 mmol) EDAC.HCl were added in this order. The reaction was monitored by TLC (EtOAc/MeOH 8:2). After 5 h, no amine starting material was detected. The mixture was evaporated to dryness under vacuum. The resulting residue was dissolved in 100 mL EtOAc. The organic solution was washed with water (2×50 mL) and brine (50 mL), dried over Na_2SO_4 and filtered. The solvent was evaporated at reduced pressure to give the raw product, which was purified by flash chromatography (*n*-hexane/EtOAc 6:4). 8.0 g of 6 as white waxy solid were obtained (yield 83%). TLC (*n*-hexane/EtOAc 1:1): R_f 0.35. HPLC: t_R 5.1 min. ^{1}H NMR ($CDCl_3$): δ 7.45–7.25 (m, 5H, aromatic H); 5.35 (s, 1H, *H*CH=), 5.30 (s, 1H, HC*H*=), 4.57 (dd, J = 8.6, 8.6 Hz, 1H, C*H*CO_2CH_3); 4.03 (m, 1H, *H*CHN); 3.74 (s, 3H, CH_3O); 3.55 (m, 2H, C*H*SPh, HC*H*N); 2.68 (m, 1H, *H*CHCHCO_2CH_3); 2.03 (m, 1H, HC*H*CHCO_2CH_3); 1.93 (s, 3H, CH_3C=). MS (EI) m/z 305 $[M]^+$. Elemental analysis: $C_{16}H_{19}NO_3S$ (305.39): calcd C 62.93, H 6.27, N 4.59; found C 63.08, H 6.40, N 4.47.

3.1.4. (4*S*)-1-[(*S*)-3-Benzoylthio-2-methylpropanoyl]-4-phenylthio-L-proline methyl ester [(*S,S,S*)]-7); (4*S*)-1-[(*R*)-3-benzoylthio-2-methylpropanoyl]-4-phenylthio-L-proline methyl ester [(*R,S,S*)]-7). 5.0 g (16 mmol) of (4*S*)-1-(2-methylpropanoyl)-4-phenylthio-L-proline methyl ester (**6**) was dissolved in 10 mL (82 mmol) thiobenzoic acid. 5 mL (29 mmol) DIPEA was added. The mixture was heated to 60 °C. The reaction was monitored by TLC (*n*-hexane/EtOAc 1:1). After 5 min, no starting material was detected. Then the reaction mixture was diluted with 50 mL of diethyl ether. The resulting solution was washed with water (50 mL), 1 N $NaHCO_3$ (50 mL), water (25 mL) and brine (25 mL). The solvent was removed under vacuum. The two diastereomers in the raw product were separated by flash chromatography (*n*-hexane/EtOAc 65:35). 3.0 g of each diastereomer were recovered (overall yield 85%). [(*S,S,S*)]-**7**. TLC (*n*-hexane/EtOAc 1:1): R_f 0.42. HPLC: t_R 11.0 min. ^{1}H NMR ($CDCl_3$): δ 8.0–7.2 (m, 10H, aromatic H); 4.53 (dd, J = 8.0, 8.0 Hz, 1H; C*H*CO_2CH_3), 4.10 (dd, J = 10.1, 6.9 Hz, 1H; *H*CHN); 3.74 (s, 3H, CH_3O); 3.72 (m, 1H, C*H*SPh); 3.49 (dd, J = 10.1, 8.5 Hz, 1H, HC*H*N); 3.22 (dd, J = 13.4, 8.0 Hz, 1H, *H*CHSCOPh); 3.15 (dd, J = 13.4, 6.1 Hz, 1H, HC*H*SCOPh); 2.88 (m, 1H, C*H*CH3); 2.64 (m, 1H, *H*CHCHCO_2CH_3); 2.01 (ddd, J = 13.0, 9.2, 7.7 Hz, 1H, HC*H*CHCO_2CH_3); 1.30 (d, J = 6.9 Hz, 3H, CH_3CH). MS (FAB) m/z 444 $[MH]^+$. Elemental analysis: $C_{23}H_{25}NO_4S_2$ (443.57): calcd C 62.28, H 5.68, N 3.16; found C 62.32, H 5.75, N 3.09. [(R,S,S)]-**7**. TLC (*n*-hexane/AcOEt 1:1): R_f 0.36. HPLC: t_R 10.2 min. ^{1}H NMR ($CDCl_3$): δ 8.0–7.2 (m, 10H, aromatic H); 4.50 (dd, J = 8.0, 8.0 Hz, 1H; C*H*CO_2CH_3), 3.97 (dd, J = 12.9, 7.2 Hz, 1H, *H*CHN); 3.75 (s, 3H, CH_3O); 3.72 (m, 2H, C*H*SPh, HC*H*N); 3.37 (dd, J = 13.4, 7.3 Hz, 1H, *H*CHSCOPh); 3.11 (dd, J = 13.4, 6.8 Hz, 1H, HC*H*SCOPh); 2.87 (m, 1H, C*H*CH_3); 2.65 (m, 1H, *H*CHCHCO_2CH_3); 2.01 (m, 1H, HC*H*CHCO_2CH_3); 1.22 (d, J = 6.9 Hz, 3H, CH_3CH).MS (FAB) m/z 444 $[MH]^+$. Elemental analysis: $C_{23}H_{25}NO_4S_2$ (443.57): calcd C 62.28, H 5.68, N 3.16; found C 62.35, H 5.76, N 3.07.

3.1.5. (4*S*)-1-[(*S*)-3-Mercapto-2-methylpropanoyl]-4-phenylthio-L-proline (Zofenoprilat 2). g (2.3 mmol) (4*S*)-1-[(*S*)-3-benzoylthio-2-methylpropanoyl]-4-phenylthio-L-proline methyl ester [(*S,S,S*)-**7**] was dissolved in 10 mL of methanol. 15 mL (15 mmol) 1 N NaOH was added. The resulting mixture was heated to 50 °C. The reaction was monitored both by TLC (*n*-hexane/EtOAc 1:1, to detect the starting material) and HPLC. After 10 min, the reaction was complete. Methanol was removed under vacuum, 2.5 N HCl was added to pH 2 and the aqueous solution was extracted with CH_2Cl_2 (3×5 mL). The combined organic extracts were washed with brine (5 mL) dried over Na_2SO_4 and filtered. The solvent was removed. 935 mg of product **2** (mixture with equimolar amount of benzoic acid) were obtained as white powder (yield 91%). HPLC: t_R 4.2 min (benzoic acid t_R 3.6

min). A sample (30 mg) was dissolved in MeOH/0.05 M KH_2PO_4 pH 3, 50:50 v/v (1 mL) and purified by semi-preparative HPLC (**2**, t_R 11.4 min; benzoic acid, t_R 5.1 min.). The fraction containing **2** was collected in water (20 mL). The product was recovered by SPE on Sep-Pak tC18 plus cartridge (Waters, 400 mg). This was washed with water (10 mL), then **2** was eluted with MeOH (2 mL). The resulting solution was dried under vacuum to give 20 mg with no detectable benzoic acid. ^{1}H NMR ($CDCl_3$): δ 7.5–7.3 (m, 5H, aromatic H); 4.50 (dd, J = 7.8, 7.8 Hz, 1H, C*H*CO_2H), 4.37 (dd, J = 10.4, 7.0 Hz, 1H, *H*CHN); 3.89 (dddd, J = 8.7, 8.7, 7.0, 5.2 Hz, 1H, C*H*SPh); 3.47 (dd, J = 10.4, 8.7 Hz, 1H, HC*H*N); 2.9–2.6 (m, 3H, CH_2SH, C*H*CH_3); 2.47 (ddd, J = 13.0, 7.8, 5.2 Hz, 1H, *H*CHCHCO_2H); 1.98 (ddd, J = 13.0, 8.7, 7.8 Hz, 1H, HC*H*CHCO_2H); 1.14 (d, J = 6.6 Hz, 3H, CH_3CH). MS (FAB) m/z 326 $[MH]^+$. Elemental analysis: $C_{15}H_{19}NO_3S_2$ (325.44): calcd C 55.36, H 5.88, N 4.30; found C 55.32, H 5.81, N 4.39. $[\alpha]_D^{20}$ (as sodium salt) = −63° (*c* 1, 0.05 N HCl in MeOH), mp 84–89 °C (as sodium salt).

3.1.6. (4*S*)-1-[(*R*)-3-Mercapto-2-methylpropanoyl]-4-phenylthio-L-proline (R,S,S of 2). Obtained from (4*S*)-1-[(*R*)-3-benzoylthio-2-methylpropanoyl]-4-phenylthio-L-proline methyl ester [(*R,S,S*)-**13**] as described for Zofenoprilat (**2**). HPLC: t_R 4.2 min (benzoic acid t_R 3.6 min). ^{1}H NMR ($CDCl_3$): δ 7.6–7.3 (m, 5H, aromatic H); 4.45 (dd, J = 7.8, 7.8 Hz, 1H, C*H*CO_2H), 4.08 (dd, J = 10.4, 7.0 Hz, 1H, *H*CHN); 3.89 (m, 1H, C*H*SPh); 3.65 (dd, J = 10.4, 8.7 Hz, 1H, HC*H*N); 3.0–2.6 (m, 3H, CH_2SH, C*H*CH_3); 2.35 (m, 1H, *H*CHCHCO_2H); 1.95 (m, 1H, HC*H*CHCO_2H); 1.12 (d, J = 6.6 Hz, 3H, CH_3CH). MS (EI) m/z 325 $[M]^+$. Elemental analysis: $C_{15}H_{19}NO_3S_2$ (325.44): calcd C 55.36, H 5.88, N 4.30; found C 55.29, H 5.79, N 4.38.

Compound **2** was converted to its sodium salt by the following procedure: it was dissolved in a mixture of methanol–water and the pH was adjusted to 7 with 0.5 N NaOH. Methanol was evaporated under vacuum and the resulting solution was lyophilised to afford a white powder. $[\alpha]_D^{20}$ = −25° (*c* 1, 0.05 N HCl in MeOH), mp 94–100 °C. ^{1}H NMR (CD_3OD): presence of two rotamers: major rotamer: δ 7.5–7.2 (m, 5H, aromatic H); 4.75 (broad, 1H, S*H*); 4.52 (dd, J = 8.3, 7.9 Hz, 1H C*H*CO_2Na); 4.22 (ddd, J = 11.8, 7.0, 1.4 Hz, 1H, *H*CHN); 3.62 (dddd, J = 10.1, 9.5, 7.3, 7.0 Hz, 1H, C*H*SPh); 3.32 (dd, J = 11.8, 9.6 Hz, 1H, HC*H*N); 2.76 (dddd, J = 12.9, 7.9, 7.3, 1.4 Hz, 1H, *H*CHCHCO_2Na); 2.73 (ddq, J = 9.4, 5.2, 6.5 Hz, 1H, C*H*CH_3); 2.65 (dd, J = 13.1, 9.4 Hz, 1H, *H*CHSH); 2.47 (dd, J = 13.1, 5.2 Hz, 1H, HC*H*SH); 2.07 (ddd, J = 12.9, 10.1, 8.3 Hz, 1H, HC*H*CHCO_2Na); 1.14 (d, J = 6.5 Hz, 3H, CH_3); minor rotamer: 7.5–7.2 (m, 5H, aromatic H); 4.75 (broad, 1H, S*H*); 4.30 (dd, J = 8.9, 8.0 Hz, 1H, C*H*CO_2Na); 4.03 (ddd, J = 10.2, 6.6, 1.4 Hz, 1H, *H*CHN); 3.72 (dddd, J = 10.7, 9.5, 7.2, 6.6 Hz, 1H, C*H*SPh); 3.62 (dd, J = 10.2, 9.5 Hz, 1H, HC*H*N); 2.87 (dd, J = 13.2, 7.1 Hz, 1H, *H*CHSH); 2.78 (ddq, J = 7.1, 6.2, 6.7 Hz, 1H, C*H*CH_3); 2.72 (dddd, J = 12.7, 8.0, 7.2, 1.4 Hz, 1H, *H*CHCHCO_2Na); 2.36 (dd, J = 13.2, 6.2 Hz, 1H, HC*H*SH); 1.91 (ddd, J = 12.7, 10.7, 8.9 Hz, 1H, HC*H*CHCO_2Na); 1.11 (d, J = 6.7 Hz, 3H, CH_3). ^{13}C NMR (CD_3OD) of the major rotamer: δ 176.6 (C); 167.9 (C); 136.1 (C); 132.7 (2 CH), 130.2 (2 CH); 128.3 (CH); 63.5 (CH); 54.1 (CH_2); 43.9 (CH); 43.4 (CH_2); 39.5 (CH_2); 38.7 (CH); 17.4 (CH_3).

3.1.7. One-pot synthesis of (4*S*)-1-[(*S*)-3-mercapto-2-methylpropanoyl]-4-phenylthio-L-proline, 2. 1 mL of magnesium bromide methacrylate solution (=0.5 M, 0.50 mmol) (**4**) was evaporated in 2 min with N_2 stream. To the resulting residue a solution of 150 mg (0.62 mmol) of (*S*)-4-(phenylthio)-L-proline methyl ester (**5**) and 130 μL (0.74 mmol) of DIPEA in 500 μL of DMF was added. Then 210 mg (0.55 mmol) of HATU was poured into the mixture. The suspension was heated to 55 °C under stirring. After 5 min a clear solution of the methacrylamide **6** was obtained. 500 μL (4.0 mmol) of thiobenzoic acid was added. The resulting solution was heated to 95 °C for 10 min to afford an equimolar mixture of the two diastereomers **7**. An amount of this solution (500 μL, containing about 5 mg of pure **7** as assessed by HPLC analysis) was diluted with 1 mL acqueous 10% $NaHCO_3$ and set aside for 30 s while a white solid (dibenzoyl disulfide) precipitated. The solution was separated by suction with a syringe and injected into a Sep-Pak tC18 Plus cartridge (Waters, 400 mg) previously activated by washing with methanol (5 mL), water (5 mL) and 10% $NaHCO_3$ (5 mL). Then the cartridge was flushed with 10% $NaHCO_3$ (5 mL) and water (5 mL) to remove thiobenzoic acid. The products **7** were recovered by washing the cartridge with 1,4-dioxane (1.8 mL).

The organic solution of **7** was poured into 1 N NaOH (1.3 mL). The resulting mixture was heated to 50 °C for 5 min. to give **2**, then cooled to rt and acidified to pH 2 by adding 4 M H_3PO_4 (400 μL). The solution was diluted with 10 mL of 0.1 M KH_2PO_4 (pH 7.5) and transferred into a new Sep-Pak cartridge. This was washed with abundant water (10 mL) and the product was eluted with MeOH (0.5 mL). The methanolic solution was evaporated under vacuum to give 2.3 mg of **2** [equimolar mixture with its (*R,S,S*) diastereomer] (overall yield 46%). HPLC t_R 4.2 min. The overall process took less than 40 min. MS (FAB) m/z 326 $[MH]^+$.

3.2. Radiosynthesis

3.2.1. [^{11}C]-(4*S*)-1-(2-methylpropenoyl)-4-phenylthio-L-proline methyl ester, [^{11}C]6. The procedures described by McCarron[12] for the preparation of [*carbonyl*-^{11}C]cyclohexanecarbonyl chloride were applied in the radiosynthesis 2-methyl-[1-^{11}C]acryloyl chloride (**8**) using isopropenylmagnesium bromide as Grignard reagent. Isopropenylmagnesium bromide (**3**) (1–2 mL, 0.5 M in THF) was passed at room temperature through a coil of fluorinated polypropylene tube (FEP) (80 cm length; i.d. 0.50 mm; o.d. 1/16′) previously flushed with argon, to leave a thin film on the inner tube surface. [^{11}C]Carbon dioxide was then dispensed at room temperature from the stainless steel loop of the Nuclear Interface module through the FEP tube in a stream of argon of 2 mL/min for 1.30 min to make the on-line formation of [^{11}C]**4.**

Thionyl chloride (3 μL, 41 μmol) in THF (400 μL) was then washed through the tube into a reactor vessel containing (*S*)-4-phenylthio-L-proline methyl ester (**5**) (3.5 mg, 15 μmol) and DIPEA (25 μL, 144 μmol) dissolved in 50 μL of THF, at 50 °C. After raising the temperature to 75 °C, the reaction mixture reacted for 5 min and [^{11}C]**6** was obtained with a radiochemical yield of 55%.

Quality control of **8** and [^{11}C]**6** were performed by an analytical HPLC (Shandon Hypersil BDS C18, 5 μ, 250×4.6 mm; CH_3CN/50 mM NaH_2PO_4 buffer pH 3, 50/50 v/v; 1 mL/min).

3.2.2. [^{11}C]-(4*S*)-1-[(*R,S*)-3-benzoylthio-2-methylpropanoyl]-4-phenylthio-L-proline methyl ester, [^{11}C]7. Thiobenzoic acid (150 μL, 1.27 mmol, diluted with 50 μL THF) was added at room temperature to the cooled reaction mixture containing [^{11}C]**6**. The mixture was heated (95 °C, 10 min), diluted with 1.5 mL of mobile phase and injected onto a semi-preparative reversed phase column [Shandon Hypersil BDS C-18 (250×10 mm, 5 μm)]. Purification of the intermediate [^{11}C]**7** was accomplished using CH_3CN/50 mM NaH_2PO_4 buffer pH 7.2, 55/45 v/v plus 0.2% TFA as mobile phase at 8 mL/min flow rate. Quality control was performed using a Shandon Hypersil BDS C-18 (250×4.6 mm, 5 μm) column with CH_3CN/50 mM NaH_2PO_4 buffer pH 3, 65/35 v/v at 1.3 mL/min flow rate.

3.2.3. [^{11}C]-(4*S*)-1-[(*R,S*)-3-mercapto-2-methylpropanoyl]-4-phenylthio-L-proline, [^{11}C]2. The effluent from the column corresponding to [^{11}C]**7** was collected in 40 mL of sterile water and recovered by SPE on a Sep-Pak tC18 plus cartridge (Millipore) pre-activated with 5 mL of ethanol followed by 20 mL of water. The Sep-Pak was washed with water (10 mL) before eluting [^{11}C]**7** with ethanol (1 mL) into a sealed vial flushed by argon. Hydrolysis proceeded by the addition of 1.2 mL of a solution of 1 N NaOH and heating at 60 °C for 8 min and was quenched by addition of a solution of 1 N HCl (1.3 mL). After cleaning the necessary parts of the module, the solution was transferred manually with a syringe containing 10 mL of a solution of NaH_2PO_4 (0.1 M; pH 7.3) into a new Sep-Pak tC18 cartridge (Waters). The Sep-Pak was washed with water (10 mL) and [^{11}C]**2** was eluted with ethanol (0.5 mL) under stream of argon into a vial containing 9.5 mL of saline solution, which was sterilised through a sterile 0.22 μm filter (Gelman Acrodisc). The pH of the final solution was neutral.

The final solution of known volume of [^{11}C]**2** and its (*R,S,S*) diasteromer was assayed for total radioactivity and a 20-μL aliquot was applied to an analytical HPLC Shandon Hypersil BDS C-18 (250×4.6 mm, 5 μm) column using CH_3CN/50 mM NaH_2PO_4 buffer pH3, 50/50 v/v as mobile phase at 1 mL/min flow rate. The amount of carrier was calculated from the UV absorbance peak area by means of the external standard calibration plot.

3.2.4. Carrier-added radiosyntheses for identification of [^{11}C]7, [^{11}C]2. The radiosynthesis was carried out as described above for the no-carried-added preparation with the addition of cold CO_2 carrier to the [^{11}C]CO_2 used for the [^{11}C]acyl chloride formation. After semi-preparative HPLC purification, the fraction corresponding to the desired ^{11}C-product was collected in 40 mL sterile water and the product was recovered by SPE on pre-activated Sep-Pak tC18 cartridge by elution with 1 mL of methanol. The non-radioactive material with the ^{11}C-labelled product was ensured by the strong UV peak at the retention time of the target compound. The peak was collected and its identity was confirmed by mass spectrometry.

[^{11}C]**7**: The APCI mass spectrum displayed an intense ion at m/z 444 corresponding to the protonated molecular ion $[M+H]^+$. Identity of the compound was ensured by its skimmer-CID spectrum (+25V), which shows ions at m/z 412 (M-CH_3O), ions at m/z 384 ($M-COOCH_3$) and at m/z 207 [$M-PheCOSCH_2CH(CH_3)CO$].

[^{11}C]**2**: the LC-APCI chromatogram of the reaction mixture showed three main peaks at t_R 3.34, 6.65 and of 9.08 min. The Mass spectrum of the first prominent peak displayed an intense ion at m/z 326 corresponding to the protonated molecular ion $[M+H]^+$ of Zofenoprilat while the spectra of the other two peaks showed ions at m/z 340 and at 354. The former compound with M_r 339 could be the methyl ester of [^{11}C]**2** originating from its incomplete hydrolysis, while the latter with M_r 353 could be either the methyl thioether and carboxymethyl ester or the ethyl ester of [^{11}C]**2** originating from a transesterification reaction with ethanol.

Addition of authentic standard to the mixture before HPLC analysis caused the expected increase in the UV peak area coeluting with the radioactive material.

3.3. PET study

A 46 year old male subject with a diagnosis of moderate hypertension (95/150 mmHg) was recruited in the nephrology department of the San Raffaele Hospital. Before his participation, the patient signed a written informed consent according to the study protocol previously approved by the Ethical Committee of the Scientific Institute H San Raffaele. A complete physical examination, blood and urine laboratory routine tests and ECG were performed at the time of recruitment. The patient suspended ACE inhibitor intake 24 h before the PET scan (5 drug half-lives). The PET study was performed with an eighteen-ring (GE Advance; General Electric Medical System, Milwaukee, WI, USA) tomograph.

The subject was positioned on the scanner bed with arms immobilized and the thorax centred in the scanner ring. Before positioning, two catheters were inserted bi-laterally in brachial veins (one for for tracer injection and one for venous blood sampling). One 15-min transmission scan was carried out with an external ^{68}Ge ring source. At the end of the transmission scan, the subject received an intravenous injection of approximately 4 mCi (148 MBq) of [^{11}C]Zofenoprilat in 5 mL of saline. After tracer injection, simultaneous

acquisition of data from 35 transaxial slices (slice thickness 4.25 mm; axial field of view 15.5 cm) was acquired according to the following schedule: six scans of 0.5 min each, two scans of 1 min each, two scans of 2.5 min each, two scans of 5 min each, two scans of 10 min and finally a 20-min scan (total acquisition time: 1 h). For the evaluation of [^{11}C]Zofenoprilat kinetics in blood, serial samples (1 mL each) were collected, from the brachial vein contra-lateral to the tracer injection site at 0.1, 1, 1.5, 3, 5, 11, 30 and 60 min after injection. Radioactivity concentration in the blood and plasma was measured by a gamma counter (LKB 1282 Compugamma CS) and expressed as KBq/mL or percentage of injected dose per mL of blood or plasma (%I.D./mL).

3.4. Data analysis

Irregular regions of interest (ROIs) were defined on the left myocardial wall, left ventricular cavity, lungs, liver, gall bladder and kidney (cortical aspect). ROIs were drawn on the temporal frame with the maximum uptake and then copied on the whole dynamic series to calculate the time–activity curve of radioactivity concentration in the various tissues examined. The external contour of myocardial wall was defined on the transmission images.

All data were corrected for the radioactive decay of carbon-11 and expressed in KBq/mL or percentage of the injected dose per mL of tissue (%I.D./mL). Radioactivity concentrations may be transformed in pmol/mL using the specific radioactivity of the tracer calculated at the time of injection.

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