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Research Article

Synthesis of Brostallicin (PNU-166196A) Labelled with ^{2}H and ^{14}C

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Summary

Brostallicin (PNU-166196A), a DNA minor groove binder, has been labelled with ^{2}H and ^{14}C. The preparation of the deuterium specifically labelled [$^{2}H_4$]brostallicin was achieved according to a nine-step sequence starting from 1,2-diamino[1,1,2,2-$^{2}H_4$]ethane **(1)**. [^{14}C]Brostallicin was obtained *via* a four-step procedure in 31% overall radiochemical yield starting from 1-methyl-4-nitropyrrole-2-[^{14}C]carboxylic acid **(9)**.

Key Words: brostallicin; PNU-166196A; distamycin; minor groove binder; deuterium; Carbon-14

Introduction

Brostallicin (PNU-166196A ; *N*-[5-[[[5-[[[2-[(aminoiminomethyl)amino]ethyl]amino]carbonyl]-1-methyl-1H-pyrrol-3-yl]amino]carbonyl]-1-methyl-1H-pyrrol-3-yl]-4-[[[4-[(2-bromo-1-oxo-2-propenyl)amino]-1-methyl-1H-pyrrol-2-yl]carbonyl]amino]-1-methyl-1H-pyrrole-2-carboxamide hydrochloride) is a new synthetic α-bromoacrylic derivative of a distamycin-like structure with four pyrrolecarbamoyl units ending with a guanidino moiety. The compound is a cytotoxic agent binding to the minor groove of DNA.

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Brostallicin was selected for clinical development for its outstanding preclinical characteristics such as *in vivo* potency and *in vitro* cytotoxicity against both murine and human cells, potent induction of apoptosis and favorable toxicity profile.[1] Moreover, *in vitro* and *in vivo* preclinical studies showed that the antitumor activity of brostallicin is increased in the presence of high levels of glutathione (GSH) and glutathione transferase (GST). Since many papers report that cellular GSH/GST levels cause the decrease of the activity of anticancer drugs (e.g. alkylating agents, anthracyclines etc.),[2] these findings might indicate a theoretical potential efficacy against tumors not responsive to conventional cytotoxic antitumor agents. Brostallicin is at present under Phase II clinical development. In order to fully investigate the absorption, distribution, metabolism, excretion and the mechanism of action of the compound, the preparation of brostallicin specifically labelled with ^{14}C was required. In addition a stable labelled version was needed to be used as internal standard to develop and validate a reliable liquid chromatography–mass spectrometry (LC–MS) assay. In the present paper the preparation of both the ^{2}H and ^{14}C labelled forms of brostallicin are reported.

Discussion and results

Although the use of a large excess of ethylenediamine (4–8 fold molar excess) would result in a considerable reduction of chemical yield, the intermediates availability, the simplicity of the method and the low cost of the ^{2}H-labelled ethylenediamine, prompted us to prepare [$^{2}H_4$]brostallicin according to the procedure shown in Scheme 1.[3] The treatment of 1,2-diamino[1,1,2,2-$^{2}H_4$]ethane **(1)** with di-tert-butyl dicarbonate in anhydrous dioxane at room temperature for 24 h afforded the corresponding Boc mono-protected intermediate **2** . The reaction of **2** with *O*-methylisourea hydrogensulfate in the presence of triethylamine in water at room temperature for 22 h, yielded the Boc protected guanidine **3**. The removal of the Boc protecting group was accomplished by treatment of **3** with a solution of 3 M HCl in methanol at room temperature for 2 h. The obtained intermediate **4** was reacted with 1-methyl-4-nitropyrrole-2-carboxylic acid chloride in the presence of $NaHCO_3$ in dioxane at room temperature for 1 h yielding the intermediate **5**. The hydrogenation over 10% Pd/C catalyst in the presence of 1 N HCl in water at room temperature for about 3 h of the

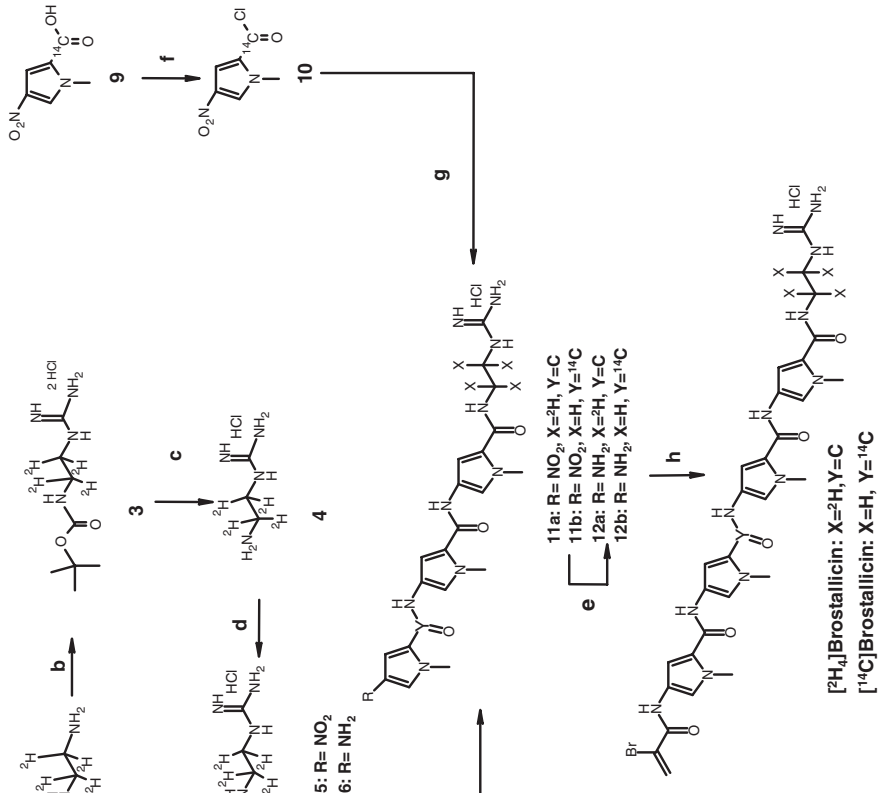
compound **5** afforded the corresponding amino derivative **6**. The addition of two more 1-methyl-4-nitropyrrole-2-carboxylic groups was performed by repeating twice the procedures described above to obtain **6** from **4**. The reaction of the amino derivative **12a** with 1-methyl-4-(α-bromo-acrylamido)pyrrole-2-carboxylic acid chloride in the presence of $NaHCO_3$ in dioxane at 0°C for 30 min, gave the crude ^{2}H-labelled brostallicin. After purification by flash-chromatography and preparative high-performance liquid chromatography (HPLC), [$^2H_4$]brostallicin was obtained in >99% chemically pure form and an isotopic enrichment of 98%. This compound was suitable for use as internal standard in a LC–MS bioanalytical assay.

As regards the ^{14}C-labelled version, the pyrrolecarbamoyl group seemed at first glance the most convenient moiety to introduce ^{14}C in brostallicin. In fact preliminary studies indicated that this unit should be stable to metabolic attack both *in vitro* and *in vivo*. Moreover, starting from 1-methyl-4-nitropyrrole-2-[^{14}C]carboxylic acid **(9)**, up to four ^{14}C atoms might be introduced in the molecular structure as needed. Another point that made this choice particularly attractive was the possibility to apply a synthetic procedure very similar to that one successfully developed for the preparation of ^{2}H brostallicin. As a compound with a specific activity more than about 1.9 GBq/mmol was not required at this stage, the most convenient approach from a radiosynthetic point of view was to label brostallicin in the pyrrolecarbamoyl unit leading to the target ^{14}C-labelled version with the minimum number of synthetic steps (see Scheme 1). The reaction of **9** with thionyl chloride in anhydrous toluene in the presence of dimethylformamide at room temperature for 90 min gave the corresponding carboxylic acid chloride **10**. The following steps were performed according to a procedure similar to that one followed during the preparation of the ^{2}H-labelled version. [^{14}C]Brostallicin was obtained with a radiochemical purity of 96% and a specific activity of 1.94 GBq/mmol. The overall radiochemical yield was approximately 31% from **9**.

Experimental

General methods

1,2-Diamino[1,1,2,2-$^2H_4$]ethane **(1)** (98% isotopic enrichment) was purchased from Aldrich. 1-Methyl-4-nitropyrrole-2-[^{14}C]carboxylic acid **(9)** (specific activity: 1.94 GBq/mmol) was supplied by NEN. All



Reagents and conditions:
a. di-tert-Butyl dicarbonate, dioxane, room temp. for 24h; **b.** O-Methylisourea.H_2SO_4, CH_3OH, H_2O, TEA, room temp. for 22h; **c.**3M HCl, CH_3OH, room temp. for 2h; **d.** 1-Methyl-4-nitropyrrole-2-carboxylic acid chloride, $NaHCO_3$, H_2O, dioxane, room temp. for 1h; **e.** H_2, 10% Pd/C, 1N HCl, room temp. for 3h; **f**. $SOCl_2$, DMF, toluene, room temp. for 90 min. **g.** Intermediate **8** $NaHCO_3$, H_2O, dioxane, room temp. for 2h. **h.** 1-methyl-4-(α-bromo-acrylamido)pyrrole-2-carboxylic acid chloride, $NaHCO_3$, H_2O, dioxane, 0°C for 30'.

Scheme 1.

solvents and reagents were of analytical grade and were used without purification unless otherwise indicated. Radioactivity measurements were performed on a Tri-Carb 2100 TR liquid scintillation analyzer (Packard) using Rialuma (Lumac System) as liquid scintillation cocktail. Chemical purities were determined by HPLC using a series-200 pump (Perkin-Elmer) equipped with Perkin-Elmer series 200 solvent degasser, Jasco series AS-950 autosampler and a LC-295 UV/VIS or a LC-235 UV diode array detector (Perkin-Elmer) and Perkin-Elmer Turbochrom 4.0 software. Radiochemical purities were determined using an A-515TR radio-HPLC analyser (Packard) equipped with a 0.5 ml homogeneous cell (liquid scintillation cocktail: Ultima Flo-M (Packard); ratio to HPLC effluent: 2/1) or by radio-TLC by using a Packard Bioscan System 200 Imaging Scanner. TLC was carried out on silica gel Merck F254 plates (20×5 cm, 0.25 mm thick) in methylene chloride: methanol : water:65% formic acid 40:8:1:1.5 (v/v) (system A) and *n*-hexane: ethyle acetate 7:3 (v/v) (system B). Preparative-HPLC was performed at 25°C using a Waters Delta prep 4000 HPLC system.

tert-Butyl 2-amino[1,1,2,2-$^2H_4$]ethylcarbamate (2)

A solution of di-tert-butyl dicarbonate (850.8 mg; 3.9 mmol) in anhydrous dioxane (60 ml) was added dropwise to a solution of **1** (1 g; 15.6 mmol) in anhydrous dioxane (60 ml) in about 3 h and the mixture was stirred for 24 h at room temperature. At the end of reaction (determined by TLC; system A [see General methods]) the solvent was removed, water (40 ml) was added to the residue and the insoluble bis-substituted by-product was separated by filtration and discarded. The filtrate was transferred into a separating funnel and extracted with methylene chloride (6×10 ml). The organic phase was dried (Na_2SO_4) and, after solvent evaporation, **2** was obtained as an oil (408.7 mg; 2.52 mmol). The yield of this step was approximately 16%.

N-(2-Amino[1,1,2,2-$^2H_4$]ethyl)guanidine dihydrochloride (4)

The intermediate **2** (408.7 mg; 2.52 mmol) was dissolved in methanol (4.65 ml) then *O*-methylisourea hydrogen sulfate (785 mg; 4.56 mmol), water (4.65 ml) and triethylamine (1.2 ml; 8.82 mmol) were added and the mixture was stirred for 22 h at room temperature. At the end of reaction (determined by TLC; system A [see General methods]) the solution was evaporated to dryness. The residue was dissolved in

ethanol (5 ml), evaporated to dryness (this operation was repeated twice) then the solid was added with absolute ethanol (8.4 ml) and stirred at 0°C for 1 h. The crude compound **3** was obtained as a suspension. After filtration through a D4 sintered-glass filter, the solid was dissolved with 3 M HCl in methanol (8.5 ml) and the resulting solution was stirred for 2 h at room temperature. At the end of reaction (determined by TLC; $R_f = 0.28$ system A [see General methods]) the solvent was evaporated to dryness. The obtained residue was washed with ethanol (4×3 ml) dissolved with methanol (10 ml) and, after solvent evaporation, the intermediate **4** was obtained (279.6 mg; 1.56 mmol). The yield of this step was approximately 62%.

N-(2-{[Amino(imino)methyl]amino}[1,1,2,2-$^2H_4$]ethyl)-1-methyl-4-nitro-1H-pyrrole-2-carboxamide hydrochloride (5)

A solution of the intermediate **4** (279.6 mg; 1.56 mmol) in water (2.7 ml) was added with $NaHCO_3$ (225 mg; 2.68 mmol) and dioxane (2.4 ml) under stirring. 1-Methyl-4-nitropyrrole-2-carboxylic acid chloride (263 mg; 1.4 mmol) in dioxane (3 ml) was added dropwise to the reaction mixture. After stirring for 1 h at room temperature the reaction was completed (determined by HPLC[†] and by TLC system A [see General methods]) and the mixture was adjusted to pH 3.5–4 with 1 N HCl aqueous solution. After solvent evaporation, the residue was purified by flash chromatography on a silica gel column (20×4 cm i.d.) using a mixture methylene chloride:methanol 5 : 1 by volume as the eluting solvent system. The fractions containing the compound were combined and after solvent evaporation, the intermediate **5** was obtained (400 mg; 1.357 mmol) 98% chemically pure (by HPLC;[†] $R_t = 5.27$ min). The yield of this step was 87%.

N-(5-{[(2-{[amino(imino)methyl]amino}[1,1,2,2-$^2H_4$]ethyl)amino]carbonyl}1-methyl-1H-pyrrol-3-yl)-1-methyl-4-nitro-1H-pyrrole-2-carboxamide hydrochloride (7)

The intermediate **5** (400 mg; 1.357 mmol) was dissolved in water (13.6 ml) then a 1 N HCl aqueous solution (1.57 ml) and Pd/C 10%

[†]Symmetry C18 column (mm150 × 4.6ID, 3.5 μm, by Waters) eluting with 20 mM Na_2H-$PO_4 \cdot 12H_2O$ at pH 4.5 with H_3PO_4:CH_3CN 90:10v/v (A) and CH_3CN (B) mixtures: 2 min at 100% A; from 100% A to 65%A in 20 min; 3 min at 65% A; from 65% A to 100%A in 1 min. Flow rate: 1 ml/min. Column temperature: 25°C. Analytical wavelength: 315 nm.

(91.6 mg) were added. The solution was stirred under hydrogen gas at room temperature until the reaction was complete (about 2.5 h; determined by TLC system A [see General methods]). The suspension was filtered through a D4 sintered-glass filter. The filtrate containing **6** was added with $NaHCO_3$ (320 mg; 3.63 mmol) then a solution of 1-methyl-4-nitropyrrole-2-carboxylic acid chloride (255 mg; 1.356 mmol) in dioxane (3 ml) was added dropwise to the reaction flask. After stirring at room temperature for 1 h, the reaction was completed (determined by TLC; system A [see General methods]). The resulting yellow suspension was filtered through a D4 sintered-glass filter and the yellow solid was washed on the filter with water (3×3 ml), suspended in ethyl acetate (30 ml) then stirred for 15 min at room temperature. The suspension was filtered and the yellow solid was dissolved in methanol (10 ml). After solvent evaporation to dryness, the crude intermediate **7** was obtained (544.5 mg; 1.23 mmol) that, although only 48% chemically pure (by HPLC;[†] $R_t = 12$ min) was used without purification in the next steps.

N-(5-{[(5-{[(2-[amino(imino)methyl]amino}[1,1,2,2-$^2H_4$]ethyl) amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)-1-methyl-4-nitro-1H-pyrrole-2-carboxamide hydrochloride (11a)

The intermediate **7** (544 mg; 1.23 mmol) was dissolved in water (18 ml) then a 1 N HCl aqueous solution (2.2 ml) and Pd/C 10% (123 mg) were added. The solution was stirred under hydrogen gas at room temperature until the reaction was completed (about 3h; determined by HPLC).[†] The suspension was filtered through a D4 sintered-glass filter and the filtrate containing the crude compound **8a** was added with $NaHCO_3$ (295 mg; 3.34 mmol). A solution of 1-methyl-4-nitropyrrole-2-carboxylic acid chloride (220 mg; 1.17 mmol) in dioxane (3.6 ml) was added dropwise to the reaction flask in 10 min and the reaction mixture was stirred at room temperature for 1 h. At the end of reaction (determined by TLC; system A [see General methods]) the resulting yellow suspension was filtered through a D4 sintered-glass filter. The yellow solid was washed with water (3×3 ml) added with ethyl acetate (5 ml) then stirred for 15 min at room temperature. The suspension was filtered, the obtained yellow solid was dissolved in methanol (10 ml)

[‡]Zorbax SB18 column (mm150 × 4.6ID, 3.5 μm, by Zorbax) eluting with CH_3CN: H_2O:TFA 10:90:0.1% (A) and CH_3CN: H_2O: TFA 90:10:0.1% (B) mixtures: from 100% A to 0% A in 20 min; 5 min at 100% B; from 0% A to 100% A in 1 min. Flow rate: 1 ml/min. Column temperature: 40°C. Analytical wavelength: 254 nm.

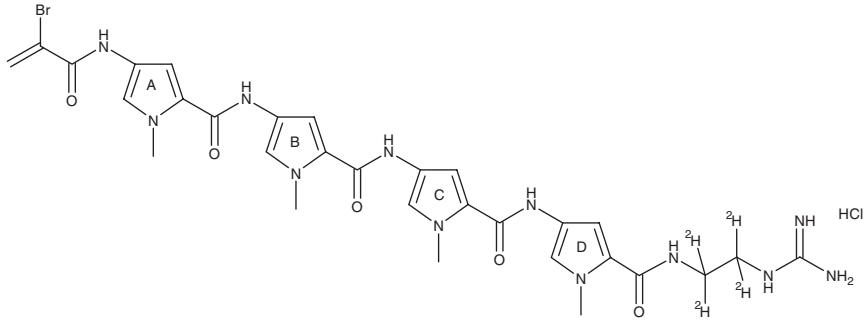
and, after solvent evaporation, the crude intermediate **11a** was obtained (155 mg; 0.287 mmol) that although only 55% chemically pure (by HPLC;† $R_t = 14$ min) was used without purification in the next steps.

N-(5-{[(5-{[(2-{[amino(imino)methyl]amino}[1,1,2,2-$^2H_4$]ethyl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)-4-[({4-[(2-bromoacryloyl)amino]-1-methyl-1H-pyrrol-2-yl}carbonyl)amino]-1-methyl-1H-pyrrole-2-carboxamide hydrochloride ([$^2H_4$]Brostallicin)

The intermediate **11a** (155 mg; 0.284 mmol) in water (6 ml) and dioxane (2 ml) was added with a 1 N HCl aqueous solution (0.37 ml) and Pd/C 10% (37 mg). The mixture of reaction was stirred under hydrogen gas at room temperature until the reaction was complete (about 3 h; determined by HPLC).† The suspension was filtered through a D4 sintered-glass filter and the filtrate containing the intermediate **12a** was added with $NaHCO_3$ (93.7 mg; 1.06 mmol). A solution of 1-methyl-4-(α-bromo-acrylamido)pyrrole-2-carboxylic acid chloride (80.7 mg; 0.32 mmol) in dioxane (1 ml) was added dropwise to the reaction flask in 15 min and the mixture was stirred at 0°C for 30 min. At the end of reaction (determined by HPLC),† the obtained yellow–brown suspension was adjusted to pH 3.5–4 with a 1 N HCl aqueous solution. After stirring for 30 min at room temperature, the suspension was evaporated to dryness, ethyl acetate (10 ml) was introduced into the flask and the yellow–brown solid residue was stirred for 10 min at room temperature. After separation by filtration, the solid residue was dissolved in methanol (10 ml) and the solvent was evaporated to dryness. The crude [$^2H_4$]brostallicin was obtained (106 mg; 0.099 mmol) in 72% chemically pure form (by HPLC;† R_t= 19.8 min).The compound was purified by flash chromatography on a silica gel column (20 × 4 cm i.d.) using a mixture of methylene chloride:methanol:water 77:20:3 by volume as eluting solvent system. [$^2H_4$]Brostallicin (32 mg) was recovered in 83% chemically pure form (by HPLC;† R_t= 19.8 min) and was further purified by preparative HPLC.§ [$^2H_4$]Brostallicin was obtained (14.7 mg; 0.019 mmol) in 99% chemically pure form (by HPLC;† $R_t = 19.8$ min). MS (ESI-MS): m/z 727 ($[MH]^+$). ^{1}H NMR (DMSO d6; 500 MHz;) δ 3.80(s, 3H, NCH_3-D); 3.83(s, 3H, NCH_3-C); 3.84(s,6H,NCH_3-

§CombiHT SB C18 column (mm150 × 21.2 ID, 5 μm, by Zorbax) eluting with CH_3CN: H_2O:TFA 10:90:0.1% (A) and CH_3CN: H_2O: TFA 90:10:0.1% (B) mixtures: isocratic at 80% A. Flow rate: 21 ml/min. Column temperature: ambient. Analytical wavelength: 315 nm.

A + NCH_3-B); 6.21(d, J = 2.8 Hz, C = CH_b H); 6.66(d, J = 2.8 Hz, C = CH_a H); 6.95(b.singlet, 1H, H-3D); 7.04(d. J = 1.9 Hz, 1H, H-3A); 7.05(b.singlet, 2H, H3-B and H3-C); 7.15(d. J = 1.9 Hz, 1H, H-5D); 7.20 (d. J = 1.9 Hz, 1H, H-5A); 7.20(b.signal, $C(NH_2) = NH_2^+$; 7.21(d. J = 1.9 Hz, 1H, H-5B); 7.22(d. J = 1.9 Hz, 1H, H-5C); 7.37(s,1H, CONH-4'); 8.03(s,1H, CONH-1'); 9.88(s,1H, CONH-D); 9.91(s,1H, CONH-C); 9.93(s,1H, CONH-B); 10.26(s,1H, CONH-A).



1-*Methyl-4-nitropyrrole-2-[^{14}C]Carboxylic Acid chloride (10)*

A solution of thionyl chloride (340 µl; 2.05 mmol) in anhydrous toluene (2 ml) was added dropwise, over a period of 10 min, to a suspension previously cooled at 0° of **9** (1.14 GBq ; 0.587 mmol) in anhydrous toluene (4 ml) and anhydrous dimethylformamide (20 µl). The reaction mixture was stirred for 90 min at room temperature. At the end of reaction (determined by radio-TLC; system B [see General methods]) the solvent was evaporated and the obtained crude compound **10** was washed with *n*-hexane (4 × 3 ml). After solvent evaporation, the intermediate **10** was obtained as a white solid (1.08 GBq; 0.556 mmol), in 95% radiochemically pure form (by radio-TLC; system B [see General methods]; $R_f = 0.41$). The yield of this step was 95%.

N-(5-{[(5-{[(2-[amino(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)-1-methyl-4-nitro-1H-pyrrole-2-[^{14}C]carboxamide hydrochloride (11b)

A solution of the compound **10** (1.08 GBq; 0.556 mmol) in dioxane (1.6 ml) was added dropwise in 10 min into a solution of **8** (281.8 mg; 0.556 mmol) in aqueous $NaHCO_3$ (pH = 8; 8 ml) and the reaction mixture was stirred at room temperature for 2 h. At the end of reaction (determined by HPLC),[‡] the resulting yellow suspension was filtered through a D4 sintered-glass filter. The yellow solid was washed with

water (3 × 3 ml), added with ethyl acetate (10 ml) then stirred for 15 min at room temperature. The suspension was filtered and the obtained yellow solid was dissolved in methanol (10 ml). After solvent evaporation, the crude intermediate **11b** was obtained (651.2 MBq; 0.334 mmol) in 85% radiochemically pure form (by HPLC;‡ R_t = 10.2 min) and used without purification in the next step. The yield of this step was approximately 60%.

N-(5-{[(5-{[(2-{[amino(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)-4-[({4-[(2-bromoacryloyl)amino]-1-methyl-1H-pyrrol-2-yl}carbonylamino]-1-methyl-1H-pyrrole-2-[^{14}C]carboxamide hydrochloride ([^{14}C]Brostallicin)

The intermediate **11b** (651.2 MBq; 0.334 mmol) in water (8 ml) and dioxane (2.5 ml) was added with a 1 N HCl aqueous solution (1.3 ml) and Pd/C 10% (71.5 mg) then the mixture was stirred under hydrogen gas at room temperature until the reaction was completed (about 4 h; determined by HPLC).‡ The suspension was filtered through a D4 sintered-glass filter and the filtrate containing the intermediate **12b** was added with $NaHCO_3$ (163 mg; 1.94 mmol). A solution of 1-methyl-4-(α-bromo-acrylamido)pyrrole-2-carboxylic acid chloride (118.33 mg; 0.406 mmol) in dioxane (1.3 ml) was added dropwise to the reaction flask in 15 min and the mixture was stirred at 0°C for 30 min. At the end of reaction (determined by HPLC)‡ the obtained yellow-brown suspension was adjusted to pH 3.5–4 with a 1 N HCl aqueous solution. After stirring for 30 min at room temperature, the suspension was filtered through a D4 sintered-glass filter. The yellow solid was washed with a mixture of water:dioxane 1:1 v:v (3 × 3 ml) added with ethyl acetate (10 ml), then stirred for 15 min at room temperature. The yellow solid was separated by filtration and dissolved with methanol (10 ml). After solvent evaporation to dryness, [^{14}C] brostallicin was obtained (358.9 MBq; 0.184 mmol) in 96% radiochemically pure form (by HPLC;‡ R_t = 11.2 min) with a specific activity of 1.94 GBq/mmol. The overall radiochemical yield was approximately 31% from **9**.

Acknowledgments

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