Synthesis of PET probe O^6-[(3-[^{11}C]methyl)benzyl]guanine by Pd^0-mediated rapid C-[^{11}C]methylation toward imaging DNA repair protein O^6-methylguanine-DNA methyltransferase in glioblastoma

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Abbreviations: O^6-methylguanine-DNA methyltransferase (MGMT), temozolomide (TMZ), O^6-benzylguanine (O^6-BG), positron emission tomography (PET), O^6-alkylguanine-DNA alkyltransferase (AGT), ^{11}C-labeled O^6-[(3-methyl)benzyl]guanine ([^{11}C]*m*MeBG), trifluoroacetyl (TFA), *tert*-butoxycarbonyl (BOC), 1,4-diazabicyclo[2.2.2]octane (DABCO)

Keywords:

^{11}C labeling

O^6-benzylguanine

Positron emission tomography probe

Rapid *C*-[^{11}C]methylation

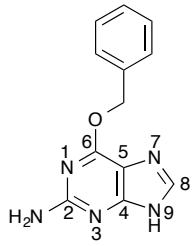
Brain cancer imaging

ABSTRACT

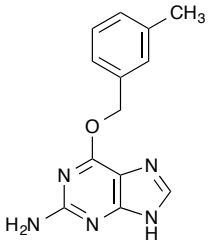
O^6-Benzylguanine (O^6-BG) is a substrate of O^6-methylguanine-DNA methyltransferase (MGMT), which is involved in drug resistance of chemotherapy in the majority of glioblastoma multiform. For clinical diagnosis, it is hoped that the MGMT expression level could be determined by a noninvasive method to understand the detailed biological properties of MGMT-specific tumors. We synthesized ^{11}C-labeled O^6-[(3-methyl)benzyl]guanine ([^{11}C]*m*MeBG) as a positron emission tomography probe. Thus, a mixed amine-protected stannyl precursor, N^9-(*tert*-butoxycarbonyl)-O^6-[3-(tributylstannyl)benzyl]-N^2-(trifluoroacetyl)guanine, was subjected to rapid *C*-[^{11}C]methylation under [^{11}C]CH_3I/[$Pd_2(dba)_3$]/P(*o*-$CH_3C_6H_4)_3$/CuCl/K_2CO_3 in NMP, followed by quick deprotection with LiOH/H_2O, giving [^{11}C]*m*MeBG with total radioactivity of 1.34 GBq and ≥99% radiochemical and chemical purities.

Epigenetic changes by aberrant promoter hypermethylation are considered to contribute significantly to tumor progression. Target genes for hypermethylation include O^6-methylguanine-DNA methyltransferase (MGMT), which encodes an important DNA-repair gene.[1] As a novel alkylation agent, temozolomide (TMZ) is currently approved for use in treatment of malignant gliomas in clinical practice.[2] TMZ undergoes spontaneous hydrolysis at physiological pH to generate 5-aminoimidazole-4-carboxamide and methyldiazonium ion, the latter of which reacts with a guanine moiety of DNA by methylation. However, the effectiveness of alkylating agents is limited by expression of MGMT. Methyl groups are removed from O^6-methylguanine-DNA by reaction with the reactive cysteine-145 thiol residue in MGMT in a suicidal manner to regenerate guanine structures in DNA.[2] A methylation-specific PCR study using human glioma cell lines showed that hypermethylation of the MGMT promoter could prevent expression of this gene with correlation to the chemosensitivity of glioma cells to TMZ.[3] For clinical diagnosis, it is hoped that the MGMT expression level could be determined by a noninvasive method to understand the detailed biological properties of MGMT-specific tumors *in vivo*.

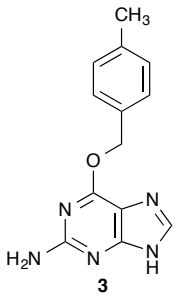
O^6-Benzylguanine (O^6-BG, **1**) is a known substrate of MGMT, which induces rapid depletion of MGMT.[4] The effectiveness of inhibitor **1** is based on the ability to irreversibly transfer its benzyl



O^6-BG (**1**)



*m*MeBG (**2**)



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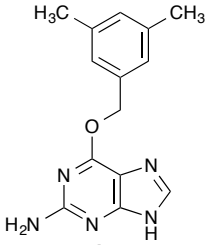

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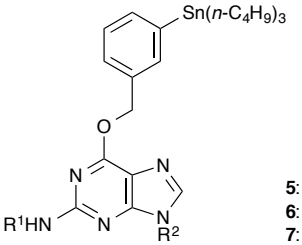


5: R^1 = H; R^2 = H.
6: R^1 = TFA; R^2 = H.
7: R^1 = TFA; R^2 = BOC.

group to a reactive cysteine residue via an S_N1 or S_N2 mechanism. Accordingly, visualization of this phenomenon in living cells using synthetic O^6-BG derivatives labeled with biotin or fluorescein revealed that the rate constant for the reaction of MGMT with O^6-BG derivatives is only 400 M^{-1} s^{-} [1,5].

Positron emission tomography (PET) is a noninvasive imaging technology with good resolution, high sensitivity, and accurate quantification. PET allows analysis of the dynamic behavior of molecules in *in vivo* systems in space and time under minute subpharmacologic doses (so-called microdoses) by using a specific molecular probe labeled with positron-emitting radionuclides, such as ^{11}C and ^{18}F.

Thus, it is considered that labeling the O^6-BG benzyl moiety with a radionuclide could be a useful PET probe for quantification of MGMT *in vivo* on a real-time basis. In this context, Zheng and co-workers first attempted to synthesize three labeled O^6-BG derivatives (O^6-[^{11}C]-[(methoxymethyl)benzyl]guanines [^{11}C]*p*-O^6-MMBG, [^{11}C]*m*-O^6-MMBG, and [^{11}C]*o*-O^6-MMBG) by simple *O*-[^{11}C]methylation using [^{11}C]methyl triflate.[6a] However, the radiochemical yields were low owing to the production of undesired N^9- and N^7-[^{11}C]methylated products. They further synthesized O^6-BG derivatives masked by methyl, benzyl, and (methoxycarbonyl)methyl groups at the N^9-position, which had similar inhibitory effects as O^6-BG

for O^6-alkylguanine-DNA alkyltransferase (AGT).[6b,c] ^{18}F labeling of O^6-BG was also performed by condensation of a 4-[^{18}F]fluorobenzyl alcohol prosthetic group with 2-aminopurine-6-yltrimethyl ammonium chloride in average decay-corrected radiochemical yield of 40%. However, this procedure required multiple radiochemical steps.

We have been intrigued by the possibility of developing new ^{11}C-labeled O^6-BG analogue by applying our rapid Pd^0-mediated cross-coupling reaction (rapid *C*-[^{11}C]methylation) between [^{11}C]methyl iodide and an organotributylstannane or organoboronic acid ester.[7,8] This method has several benefits: (1) radiolabeling could be conducted in one pot at the final stage; (2) carbon–[^{11}C]carbon bonds are metabolically much more stable than carbon–heteroatom bonds, resulting in the provision of reliable PET images; and (3) as the methyl group is the smallest nonpolar substituent, the change in biological activity would be minimized in comparison with that of the parent compound.[8] Herein, we describe the design and synthesis of ^{11}C-labeled O^6-[(3-methyl)benzyl]guanine ([^{11}C]*m*MeBG, [^{11}C]**2**) toward the imaging of MGMT-expressing brain tumors.

Three O^6-BG derivatives (O^6-[(3-methyl)benzyl]guanine (**2**), O^6-[(4-methyl)benzyl]guanine (**3**), and O^6-[(3,5-dimethyl)benzyl]guanine (**4**)) were synthesized according to a literature procedure[9] from the corresponding alcohols and 2-amino-6-chloropurine.

The MGMT enzymatic activities of compounds **2**–**4** were examined using fluorometrically labeled oligonucleotide substrates containing MGMT-specific DNA lesions and capillary electrophoresis to detect and quantify these lesions.[10] As expected, compounds **2**–**4** proved to be MGMT inhibitors with similar activities to the parent compound O^6-BG (**1**). Moreover, *meta*- and *para*-substituted derivatives **2** and **3**, were slightly more active than O^6-BG (Fig. 1).[11] A pharmacokinetic study focused on the brain permeability and metabolism in Sprague-Dawley rat by intravenous (i.v.) administration (10 mg/kg) was conducted to determine concentrations of each cold compounds **2** and **3** in the brain and plasma. Thus, tissue and blood samples collected after 30 min revealed that the 0.1% of the administered **2** (or **3**) permeated the rat brain with the brain/plasma ratio of 0.3 for both compounds (Supporting Information), judging that **2** and **3** are blood-brain barrier permeable substrate. It was also found that **2** and **3** are stable in plasma, but, after i.v. administration in rat, **2** is highly stable whereas **3** undergoes metabolization gradually to unknown more polar compounds within 2 hrs (Supporting Information). Thus, we selected **2** as the target compound for ^{11}C-labeling.

We planned to synthesize ^{11}C-labeled **2** ([^{11}C]**2**) via the corresponding stannyl precursor using rapid sp^3–sp^2(phenyl)-type Pd^0-mediated cross-coupling.[7] As it was anticipated that the reaction would be accompanied by destannylation of the stannyl substrate, which possesses an acidic proton,

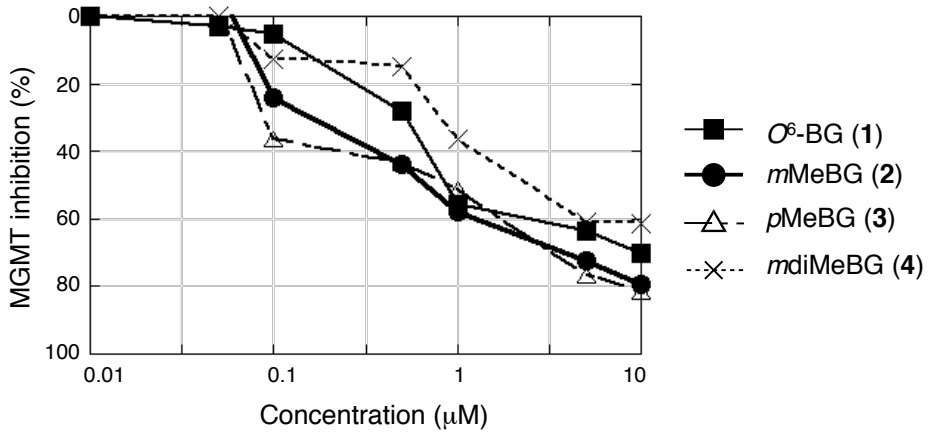
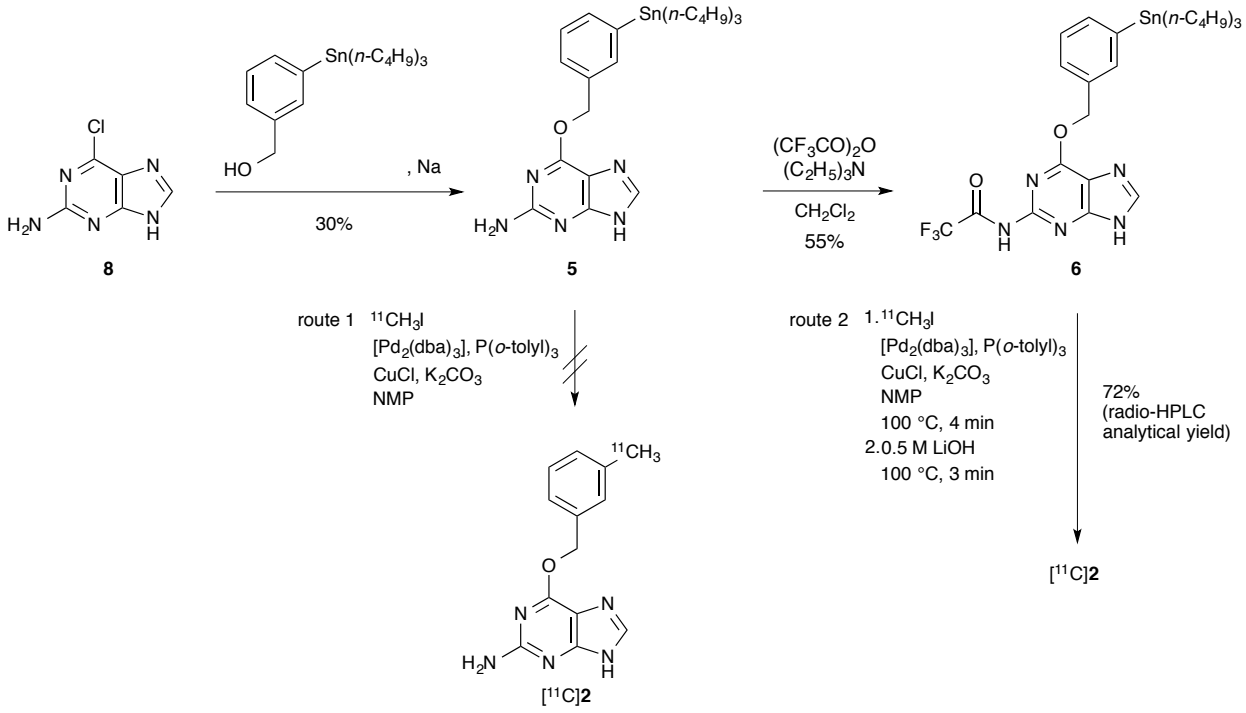
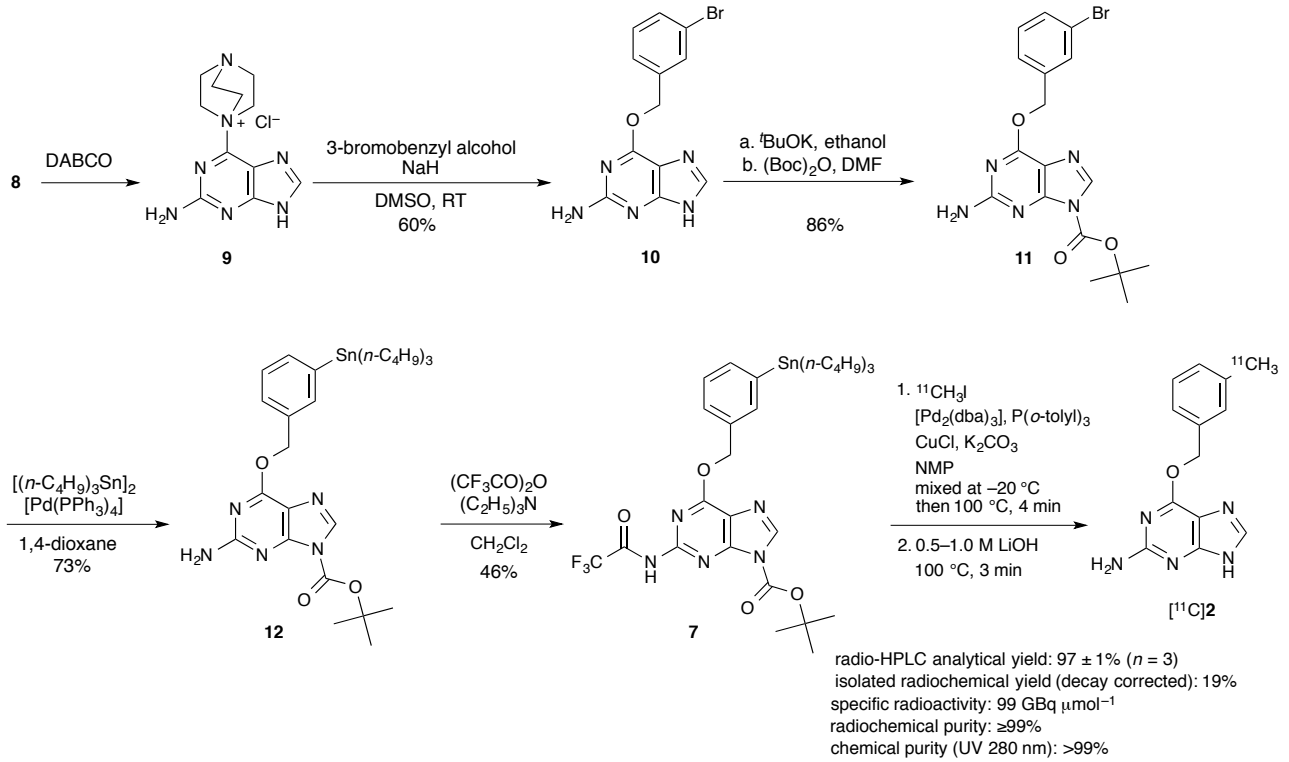


Fig. 1. MGMT inhibitory activities of O^6-benzylguanine (**1**), O^6-[(3-methyl)benzyl]guanine (**2**), O^6-[(4-methyl)benzyl]guanine (**3**), and O^6-[(3,5-dimethyl)benzyl]guanine (**4**).

we used fairly basic conditions comprising a $CuCl/K_2CO_3$ synergic system to avoid such a side reaction.[12] Thus, we first tried the coupling reaction under $[Pd_2(dba)_3]/P(o\text{-}CH_3C_6H_4)_3/CuCl/K_2CO_3$ using $[^{11}C]CH_3I$ and non-protected stannyl precursor **5**, which was prepared from the reaction of 2-amino-6-chloropurine (**8**) and 3-tributylstannylbenzyl alcohol sodium alkoxide, but the desired product was not obtained, as judged by radio-HPLC analysis (Scheme 1, route 1; see also Supporting Information Fig. S4). Accordingly, we attempted to protect the amino group and imidazole nitrogen of **5**. We first selected the trifluoroacetyl (TFA) group, for which deprotection can be achieved under basic conditions.[13] Unexpectedly, only the amino group was protected to give mono-protected **6** (55% yield, Scheme 1, route 2), even when an excess amount of trifluoroacetic anhydride was used (>3 equiv). In contrast, protection by the *tert*-butoxycarbonyl (BOC) group occurred selectively at the 9-position of the guanine moiety under treatment of **5** with 1 equiv of potassium *tert*-butoxide in ethanol followed by the addition of $(Boc)_2O$ in DMF (86% yield). Moreover, it is noted to find that the BOC group was readily removed under usual basic conditions (NaOH aq. or LiOH aq.) within a few minutes instead of the acidic conditions usually employed for BOC group removal.[14] Such valuable information enabled us to synthesis stannyl substrate **7** with mixed TFA and BOC protecting groups, as shown in Scheme 2. Thus, quaternary amine 1-(2-amino-9*H*-purin-6-yl)-4-aza-1-azoniabicyclo[2.2.2]octane chloride (**9**), prepared from **8** and 1,4-diazabicyclo[2.2.2]octane (DABCO) according to the reported procedure,[9] was reacted with an excess amount of 3-bromobenzyl alcohol deprotonated with sodium hydride to produce O^6-[(3-bromo)benzyl]guanine (**10**) in 60% yield. Then, **10** was selectively protected at the 9-position of the guanine moiety by a BOC group to give **11** in 86% yield. The reaction of **11** with hexa-*n*-butylditin in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) gave stannane **12** in 73% yield. TFA protection of amino group in the 2-position of the guanine



Scheme 1. Syntheses of non- and mono-protected stannyl precursors **5** and **6**, and attempted rapid *C*-methylations toward radiolabeled [^{11}C]*m*MeBG ([^{11}C]**2**).



Scheme 2. Synthesis of mixed di-protected stannyl precursor **7** and highly efficient synthesis of ^{11}C-labeled O^6-[(3-methyl)benzyl]guanine ([^{11}C]**2**) by sequential rapid *C*-methylation of **7** and deprotection.

moiety of **12** was performed to give N^9-(*tert*-butoxycarbonyl)-O^6-[3-(tributylstannyl)benzyl]-N^2-(trifluoroacetyl)guanine (**7**) in 46% yield.

Thus, we prepared the mono-protected and mixed di-protected stannyl precursors **6** and **7** for ^{11}C-labeling. In addition, we recently realized a one-pot protocol with high efficiency for our Pd^0-mediated rapid *C*-methylation using a stannyl precursor by introducing [^{11}C]CH_3I under bubbling with the reaction mixture at low temperature (–20 °C). The success of this approach is presumably due to the suppression of homocoupling of organocopper R-Cu generated *in situ*.[15] Thus, the actual reaction was conducted according to such a temperature-controlled one-pot method,[15] in which the reaction mixture of $[Pd_2(dba)_3]$/P(*o*-$CH_3C_6H_4)_3$[16]/CuCl/K_2CO_3 (1:10:4:10, mol ratio) and the stannyl substrate in NMP was maintained below –10 °C by cooling during [^{11}C]CH_3I preparation and further cooled at the same temperature during [^{11}C]CH_3I bubbling in the reaction mixture. Subsequently, the temperature was elevated to 100 °C and the reaction mixture was maintained at this temperature for 4 min. Deprotection was conducted by the addition of 0.5 M LiOH aq. at 100 °C for 3 min, and then the reaction mixture was diluted with the HPLC eluent containing sodium ascorbate. Thus, mono-protected stannyl substrate **6** was first subjected to improved *C*-[^{11}C]methylation conditions, followed by deprotection to give [^{11}C]**2** in 72%

radio-HPLC analytical yield (Scheme 1, route 2; see also Supporting Information Fig. S5). It was considered that additional side methylation reactions at the 7- and/or 9-positions of the guanine moiety would have decreased the yield by a considerable extent.[6a] In contrast, as shown in Scheme 2, the reaction using mixed di-protected stannane **7** was greatly improved (conditions for [^{11}C]methylation and deprotection: [$Pd_2(dba)_3$]/P(*o*-$CH_3C_6H_4)_3$/CuCl/K_2CO_3 (1:10:4:10) in NMP at 100 °C for 4 min and 0.5 M LiOH at 100 °C for 3 min), giving [^{11}C]**2** in much higher yield (97 ± 1% HPLC analytical yield, n = 3, Fig. 2).[17] The total radioactivity of [^{11}C]**2** after purification by preparative HPLC was 1.34 GBq. The decay-corrected radiochemical yield based on [^{11}C]CH_3I was 19%. The radiochemical and chemical purities of [^{11}C]**2** were ≥99% and >99%, respectively. The specific radioactivity of [^{11}C]**2** after formulation was in the range of 99 GBq μmol^{-1}. The total synthetic time including HPLC purification and formulation was 47 min. We also found that the use of sodium ascorbate as a weaker base instead of K_2CO_3 was also fairly effective for ^{11}C-labeling, giving [^{11}C]**2** in 86 ± 9% (n = 3) radio-HPLC analytical yield (see Supporting Information). As an efficient radical scavenger, sodium ascorbate could serve not only to maintain the basic reaction conditions but to prevent product decomposition induced by radiolysis during the reaction and work-up.[18] Thus, the use of sodium ascorbate has potential for ^{11}C-labeling.

In addition, the rapid methylation reaction was substantiated by using non-radioactive CH_3I.

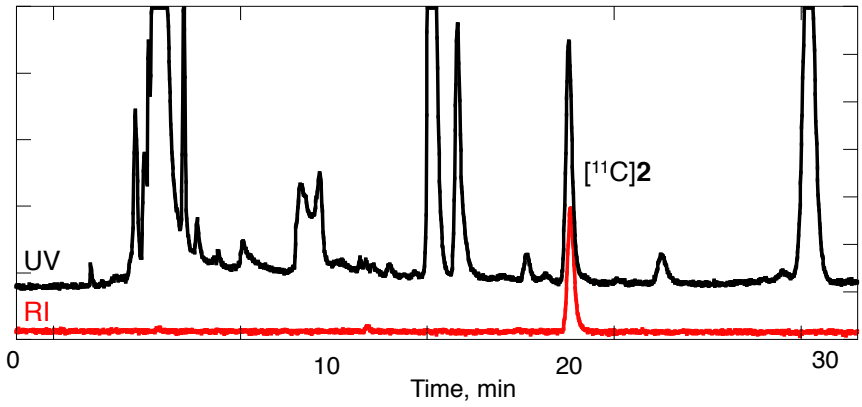


Fig. 2. Analytical HPLC chromatograms (upper: UV (280 nm); lower: RI detection) of the reaction mixture after *C*-[^{11}C]methylation using stannyl precursor **7** followed by deprotection.

Thus, the coupling reaction of CH_3I with an excess amount of **7** (10 equiv) was conducted using $[Pd_2(dba)_3]/P(o\text{-}CH_3C_6H_4)_3/CuCl/K_2CO_3$ (1:16:4:10, mol ratio) in NMP at 80 °C for 4 min, and then, the TFA and BOC groups of **7** were cleaved by heating at the same temperature for 3 min using 1.0 M LiOH aq. to give **2** in 100% yield (see Supporting Information).

In summary, ^{11}C-labeling of O^6-[(3-methyl)benzyl]guanine was accomplished efficiently by the combination of rapid Pd^0-mediated *C*-[^{11}C]methylation using [^{11}C]CH_3I and a stannyl precursor under the $CuCl/K_2CO_3$ synergic system with subsequent quick removal of the protection groups on the guanine moiety. The protocol for ^{11}C-labeling of O^6-BG can be applied to a variety of compounds with an O^6-BG moiety, such as O^4-benzylfolic acid,[19] glucose-conjugated O^6-BG MGMT inhibitors,[20] antiviral purine-β–lactam hybrids,[21] and O^6-BG derivatives as substrates of cyclin-dependent kinases, which attract considerable attention as targets for therapeutic intervention in cancer.[22] We hope that [^{11}C]**2** will serve to be an efficient PET imaging agent as the predictive marker for MGMT-expressed glioblastoma in the brain and the therapeutic effects of TMZ and related antitumor drugs. Molecular imaging studies with [^{11}C]**2** will be reported in due course.

Acknowledgements

This work was supported in part by a Grant-in Aid from JSPS KAKENHI (Grant Number (A)

25242070 (M.S.), (B) 21390409 and (A) 24249073 (T.W.), (B) 21390408 (A.N.), and (C) 50402160 (H.K.)) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. We also thank Dr. H. Doi and Ms. T. Mori (RIKEN Center for Life Science Technologies), who contributed at an early stage of the labeling study, and Mr. Y. Kawasumi (Sumitomo Heavy Industries, Ltd.) for operating the cyclotron system. We gratefully thank for Mr. M. Satoh, Dr. K. Matsuda, Dr. O. Nakayama, and Ms. M. Murai (Molecular Imaging CRO Network), who contributed to *in vivo* pharmacokinetic and metabolic stability studies of **2** and **3** using Sprague-Dawley rat. We would like to thank Editage (www.editage.jp) for English language editing.

Supplementary data

Supplementary data associated with this article can be found in the online version, at

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17. A mixture of [$Pd_2(dba)_3$] (1.0 mg, 1.1 μmol), P(*o*-tolyl)$_3$ (3.4 mg, 11 μmol), CuCl (0.4 mg, 4 μmol), and K_2CO_3 (1.4 mg, 11 μmol), and stannyl precursor **7** (0.8 mg, 1.1 μmol) in NMP (300 μL) was kept below –10 °C by cooling while waiting for the [^{11}C]CH_3I preparation. [^{11}C]CH_3I formed from [^{11}C]CO_2 using the conventional $LiAlH_4$ method was trapped into the reaction mixture, which was stopped cooling, followed by the mixture was quickly heated to 100 °C, and left to stand for 2 min, and then bubbling with N_2 gas for 2 min. The reaction mixture was cooled for 10 seconds to avoid bumping. 0.5 M LiOH aq. (600 μL) solution was added to the mixture and then it was bubbling with N_2 gas at 100 °C for 3 min. After diluting by CH_3CN/H_2O (35:65 *v*/*v*, 600 μL) containing sodium ascorbate (2.2 mg, 11 μmol), the mixture was passed through the fine filter F (F162, Forte Grow Medical co., ltd.) with quartz glass wool (Tosho co., ltd.), and injected into preparative HPLC (mobile phase, CH_3CN/20 mM sodium phosphate (pH 4.9) = 5:95 and 35:65; column, CAPCELL PAK C18 MG 120, 20 (i.d.) × 250 mm; flow rate, 10 mL/min; UV detection, 280 nm; retention time, 21 min). Peak areas on the radio-HPLC of the reaction mixture indicated a HPLC analytical yield of 97 ± 1% (*n* = 3). The desired fraction was collected into a flask, and the organic solvent was removed under the reduced pressure. The desired radiotracer was dissolved in 0.25% solution of polysorbate 80 in physiological saline (3.0 mL). The total synthesis time including HPLC purification and radiopharmaceutical formulation for intravenous administration was 47 min. The isolated radioactivity was 1.34 GBq at the end of synthesis and the specific radioactivity was 99 GBq μmol^{-1}. The decay-corrected radiochemical yield was 19%, which was calculated on the basis of the radioactivity of [^{11}C]CH_3I trapped in the solution. The chemical identity of [^{11}C]**2** was confirmed by co-injection with the authentic sample of nonradiolabeled **2** on analytical HPLC (mobile phase, CH_3CN/20 mM sodium phosphate = 40:60; column,

CAPCELL PAK C18, 4.6 (i.d.) × 250 mm; flow rate, 1 mL/min; UV detection, 280 nm; retention time, 6.1 min). The chemical purity analyzed at 280 nm and the radiochemical purity were ≥99% and >99%, respectively.

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Captions and legends

Fig. 1. MGMT inhibitory activities of O^6-benzylguanine (**1**), O^6-[(3-methyl)benzyl]guanine (**2**), O^6-[(4-methyl)benzyl]guanine (**3**), and O^6-[(3,5-dimethyl)benzyl]guanine (**4**).

Scheme 1. Syntheses of non- and mono-protected stannyl precursors **5** and **6**, and attempted rapid *C*-methylations toward radiolabeled [^{11}C]*m*MeBG ([^{11}C]**2**).

Scheme 2. Synthesis of mixed di-protected stannyl precursor **7** and highly efficient synthesis of ^{11}C-labeled O^6-[(3-methyl)benzyl]guanine ([^{11}C]**2**) by sequential rapid *C*-methylation of **7** and deprotection.

Fig. 2. Analytical HPLC chromatograms (upper: UV (280 nm); lower: RI detection) of the reaction mixture after *C*-[^{11}C]methylation using stannyl precursor **7** followed by deprotection.