Title: Tritium *O*-Methylation of *N*-Alkoxy Maleimide Derivatives as Labeling Reagents for Biomolecules

Running Title: Tritium *O*-methylation of maleimide derivatives

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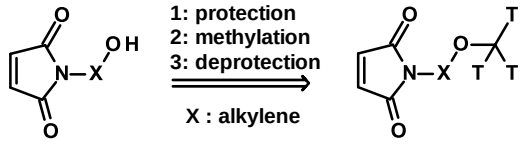
Graphical Table of Contents:



In a 3-step synthesis, *N*-substituted maleimides containing exocyclic hydroxy-functionality can be *O*-methylated under basic conditions from [^{3}H]methyl nosylate in overall radiochemical yields of 13-15%.

Abstract

An efficient procedure to access tritium-labeled maleimide derivatives in a high specific activity has been developed. *N*-Substituted maleimides containing the hydroxy functionality are *O*-methylated in a 3-step synthesis route, including 1) Diels-Alder protection of the maleimide core, 2) *O*-methylation by the use of commercially available [^{3}H]methyl nosylate, and 3) deprotection by retro-Diels-Alder reaction. With our procedure, *N*-hydroxyalkyl maleimide derivatives can be labeled in overall radiochemical yields of 13% - 15% and in > 98% radiochemical purity. The major advantage of *N*-alkoxy maleimides in comparison to *N*-alkylated maleimides such as *N*-ethylmaleimide is their lower volatility, which enables safer handling with respect to radiation-safety protection. Tritium labeled maleimide building blocks allow subsequent Michael-type conjugation reactions of thiol containing biomolecules for mechanistic *in vitro* or *in vivo* studies.

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Introduction

Conjugations with functionalized maleimide derivatives have been popular in biochemistry or biotechnology.[1] The double bond of maleimides may undergo a Michael-type addition with sulfhydryl groups to form stable thioether bonds. The main use of maleimide derivatives is the bioconjugation to thiols, which are either naturally present in biomolecules or have been previously introduced. This technique is widely used in radio- or fluorescent labeling,[2, 3] or for the preparation of antibody-drug-conjugates.[4] Thiol-maleimide additions take place quickly in the pH range of 6.5 to 7.5 in a high yield without the formation of byproducts, even in aqueous solution. Several functionalized maleimides are commercially available or accessible in a few synthesis steps. The simplest functionalization of maleimides is by formation of the equivalent *N*-alkyl derivatives.

Non-radiolabeled alkylated maleimide derivatives, such as *N*-ethylmaleimide (NEM) or *N*-methylmaleimide (NMM) are used in protein analytics, e.g. to identify free sulfhydryl groups in antibodies[5] or for thiol-blocking in cysteine residues.[6, 7] Tritium labeled alkyl-maleimides, on the other hand, are used to determine the number of sulfhydryl groups in proteins,[8] to identify surface-exposed cysteine residues,[9] or in assays of protein palmitoylation.[10] *N*-Alkylated tritium maleimide derivatives are also used in Michael-type additions to thiol-linkers in several types of biomolecules for tracking and monitoring, e.g. in oligonucleotides or proteins.[11, 12] A general disadvantage of NEM and NMM is their high volatility, which is a major challenge in dealing with them in radioactive conjugation reactions.

The most common way to access *N*-alkylated maleimides is the reaction of maleic anhydride with a primary amine, followed by dehydration.[13] However, the translation of the aforementioned synthetic strategy for the preparation of tritiated analogues becomes a challenge due to the high volatility of the required primary amines. Methylamine with its boiling point of -6 °C and ethylamine (17 °C) pose a safety risk when handling radioactive compounds. In addition to the high volatility of these alkylamines, the corresponding tritiated compounds are only commercially available with a specific molar activity in the range of 10 – 20 Ci/mmol. For this reason, an alternative and safe synthetic route was required for the introduction of a tritium label on maleimide derivatives.

Palladium-catalyzed reduction of *N*-alkylated maleimides bearing an exocyclic carbon-carbon double or triple bond in the presence of tritium gas is not a viable option, since the maleimide double bond, required for a subsequent Michael-addition, will be reduced as well. A promising concept can be designed by late-stage alkylation of nucleophiles, such as amines or alcohols.

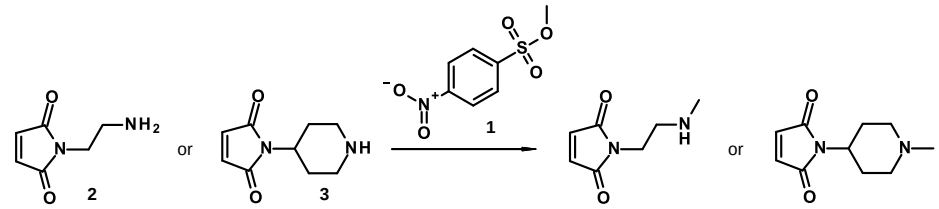
In this article, an alternative strategy to access tritium-labeled maleimide derivatives using *N*-hydroxyalkyl *exo*-maleimide-2,5-dimethylfuran cycloadducts in good yields and high specific molar activities (> 40 Ci/mmol) is described. The synthesis avoids the use of volatile radioactive reagents and therefore significantly mitigates the safety risks in the preparation of tritiated *N*-alkyl maleimides. For a safe and robust concept with regard to late-stage labeling, we have examined *N*- and *O*-methylation with methyl nosylate.

Results & Discussion

Since Pounds' report in 2004,[14] [^{3}H]methyl nosylate **1** ([^{3}H]methyl-4-nitrobenzenesulfonate) has been used in tritium-based chemistry as a radiochemically stable reagent for methylation of alcohols or amines. [^{3}H]Methyl nosylate is commercially available at a specific molar activity of > 80 Ci/mmol and in contrast to the corresponding methylation reagent [^{3}H]methyl iodide it is not volatile. The fastest and easiest way to synthesize methylated maleimide derivatives is the direct alkylation of an amine or hydroxy functionalized maleimide precursor.

Direct methylation of N-substituted maleimides containing amino and hydroxy functionalities with [^{3}H]methyl nosylate

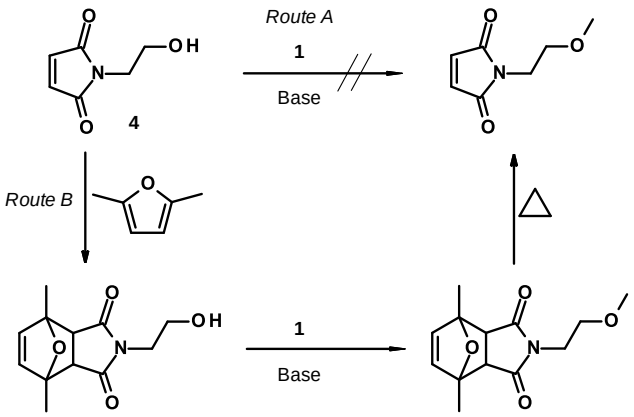
In the first experiments, it was aimed for an *N*-methylation and therefore two maleimide derivatives *N*-(2-aminoethyl)-maleimide **2** and *N*-(4-piperidyl)-maleimide **3** were selected (**Scheme 1**). Although the methylated products could be isolated in low yield but in high purity, the stability of the products in solution was low and the compounds decomposed over 24 hours. It was hypothesized for **2** that this inherent instability might originate from the unprotected amine functionality, which is prone to react intramolecularly with the electrophilic imide carbonyls or intermolecularly with the activated olefin. In addition for **2** and **3**, the basicity of the methylated amine may promote hydrolysis of the maleimide core, which leads to ring opening. This observation confirms results reported in previous articles on the hydrolysis of maleimides in alkaline solutions.[15] For this reason, the strategy was changed from *N*-methylation to *O*-methylation of a primary alcohol by using *N*-(2-hydroxyethyl)-maleimide **4** as substrate. Since a base, such as sodium hydride, sodium *tert*-butoxide, or lithium bis(trimethylsilyl)amide, was required for deprotonation of primary alcohols to perform a methylation with methyl nosylate, this synthesis route was also unsuccessful (**Scheme 2: *Route A***).



Scheme 1: *N*-Methylation of maleimide derivatives containing amine functionality.

Using Diels Alder cycloadduct as a protecting group for hydroxylethyl-maleimide prior to methylation with [^{3}H]methyl nosylate

N-(2-Hydroxyethyl)-maleimide **4** was selected as starting material to investigate the *O*-methylation of maleimide derivatives, which involves three steps: 1) protection of the maleimide core; 2) methylation on deprotonated hydroxy functionality; 3) deprotection to the final maleimide (**Scheme 2:** ***Route B***).



Scheme 2: Investigated synthesis routes to methylate *N*-(2-hydroxyethyl)-maleimide **4** from methyl nosylate **1**. *Route A*: direct methylation starting from unprotected **4** and **1** did not lead to the desired product under basic conditions. *Route B*: protected **4** can be methylated in the presence of bases and deprotected by heating to achieve the target methylated maleimide derivative.

The introduction of a protecting group is essential for a chemical modification of maleimides under basic conditions. Maleimides can generally be easily protected under mild heating with furan analogues, resulting in a [4+2] Diels-Alder (DA) cycloadduct.[16] The formed furan-maleimide cycloadduct prevents the possibility of a nucleophilic attack and allows *O*-alkylation under strongly basic conditions. Two relevant aspects are to be considered for a successful methylation labeling concept under basic conditions: 1) the selection of the furan analogue for the protection, and 2) the selection of the diastereo isomer after the [4+2]-cycloaddition for the subsequent deprotection.

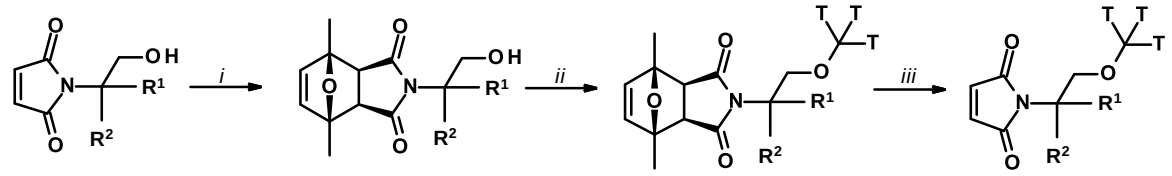
It is well known that the DA reaction between electron-deficient maleimides and dienes such as furans leads to a mixture of two diastereomers: *endo*, the kinetically controlled compound, and *exo*, the thermodynamically more stable compound.[17] Although the *endo* compound can be

deprotected more easily under retro-Diels-Alder (rDA) reaction conditions by heating,[18] it is also less stable towards a base compared to the *exo* compound.[19, 20]

A comparison of different furan derivatives as a protecting group, namely furan, 2-methylfuran and 2,5-dimethylfuran, showed that 2,5-dimethylfuran has the best stability towards bases.[21] Based on this knowledge, the synthesis route was adjusted as shown in **Scheme 3**.

After DA protection with 2,5-dimethylfuran, the obtained *exo*/*endo* intermediates formed in a diastereomeric mixture ratio of 4:1 were separated into the pure *exo* compound in 55-60% yield by the use of silica-based flash chromatography. In order to achieve a simpler handling on small scale for the methylation step, commercially available [^{3}H]methyl noslyate (**1**, 88 Ci/mmol) was diluted with non-labeled methyl nosylate to achieve a specific molar activity of 44 Ci/mmol, which comfortably still meets the requirements (> 1 Ci/mmol) for tracking of molecules (< 10,000 Da) after conjugation to tritium labeled maleimides. A solid phase supported workup was carried out by using strong ion exchange (SCX-2/SAX) cartridges.[22] This method allows a fast, safe, and efficient work-up for tritium labeled compounds with high recovery rates. Deprotection was carried out by a retro-Diels-Alder reaction by heating at 110 °C to give the desired tritium labeled *N*-[^{3}H]-2-methoxyethyl maleimide **13** in an overall radiochemical yield of 15%.

The robustness of this synthetic route was confirmed by repeating it with *N*-(2-hydroxy-1-methyl-ethyl)maleimide **5** and *N*-[1-(hydroxymethyl) cyclopropyl]maleimide **6** as starting materials. In both cases similar yields of 13%-15% could be observed.



4: $R^1 = R^2 = H$
5: $R^1 = H$, R^2 = methyl
6: R^1 and R^2 = (*)

7: $R^1 = R^2 = H$
8: $R^1 = H$, R^2 = methyl
9: R^1 and R^2 = (*)

10: $R^1 = R^2 = H$
11: $R^1 = H$, R^2 = methyl
12: R^1 and R^2 = (*)

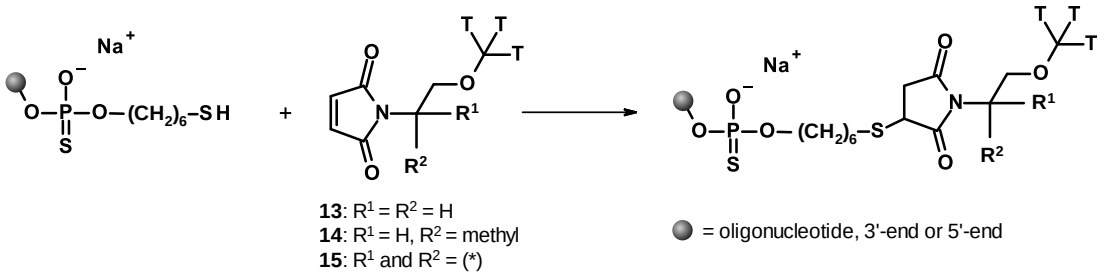
13: $R^1 = R^2 = H$
14: $R^1 = H$, R^2 = methyl
15: R^1 and R^2 = (*)

Scheme 3: Synthesis route to tritium-methylated *N*-alkoxy maleimide derivatives **13**, **14**, **15**. *i*: 2,5-dimethylfuran, acetonitrile, 65 °C, 16 h. *ii*: [^{3}H]methyl nosylate **1**, sodium *tert*-butoxide, toluene, 22 °C, 2.5 h. *iii*: toluene, 110 °C, 2 h. (*): R^1 and R^2 together with the carbon atom to which they are attached form a cyclopropyl ring.

Conjugation of [^{3}H]-Maleimides on Thiol-functionalized Oligonucleotides

To prove the reactivity of the tritium-labeled maleimides **13**, **14**, and **15**, 2 different tool-oligonucleotides were used for conjugation experiments. The oligonucleotides contain a phosphorothioate-hexylenethiol-linker, either at 5'-end or 3'-end, which can be used for a Michael-type addition (**Scheme 4**). For the conjugation experiments, the purified tritium-labeled maleimides **13**, **14**, and **15** were used directly from the preparative HPLC-fractions

without solvent exchange and added to a pH 7 buffered oligonucleotide solution. In order to achieve the highest possible conversion of tritiated maleimides, the oligonucleotide was used for the conjugation in a double molar excess, followed by a post-conjugation with the corresponding cold (non-radioactive) maleimide derivative in a 10-fold molar excess. In this way, a lower specific activity was consciously accepted. However, the resulting specific molar activities of 10 to 20 Ci/mmol still comfortable meet the requirements for *in vivo* disposition and QWBA studies. In order to determine the specific activity of the conjugates, the activity concentration [mCi/mL] of the oligonucleotide solution was determined by liquid scintillation counting and divided by the content concentration [mg/mL] measured using a spectrometer to give the specific activity in [mCi/mg]. Based on the molecular weight, the specific molar activity in [Ci/mmol] could be calculated for each tritium-labeled oligonucleotide.



Scheme 4: General conjugation reaction of thiol-functionalized oligonucleotides with maleimide derivatives **13**, **14**, **15**. (*): R^1 and R^2 together with the carbon atom to which they are attached form a cyclopropyl ring.

Conclusions

In this manuscript, we have disclosed a facile and efficient method for radiolabeling of *N*-alkoxy maleimide derivatives from [^{3}H]methyl nosylate. One of the most important features of these tritium-methyl-labeled compounds is that they are less volatile than the corresponding *N*-alkylated maleimides *N*-ethylmaleimide (NEM) or *N*-methylmaleimide (NMM). The standard molar enthalpy of sublimation of NMM ($\Delta_{sub}H^{\circ}_m$ = 73.3 KJ/mol at 293.15 K)[23] is comparable to that of naphthalene ($\Delta_{sub}H^{\circ}_m$ = 73.0 KJ/mol at 298.15 K).[24] No data could be found in the literature on NEM. However, a rapid weight loss of NEM was observed during previous conjugation preparations. Since tritium-labeled NEM is supplied in a pentane solution, a solvent exchange must be carried out prior to bioconjugations. This handling bears the risk of undesired release of radioactivity and loss of NEM.

Starting from *N*-substituted maleimides containing exocyclic hydroxy functionality, base-stable intermediates of *O*-methylations could be provided by a Diels-Alder [4+2] cycloaddition reaction in the presence of 2,5-dimethylfuran. Protected maleimides enable the methylation of primary alcohols under basic conditions and prevent nucleophilic attack from Michael-type

donors. A solid-phase supported work-up by the use of strong ion exchange cartridges (SCX-2/SAX) leads to pure intermediates. The final products were gained *via* a retro Diels-Alder reaction by heating the protected tritium-labeled maleimides. Although multi-step synthesis of tritium-labeled compounds is a particular challenge, this synthetic route is characterized by mild reaction conditions, efficient work-up, and high radiochemical purities. This is also reflected in the overall radiochemical yields of 13-15%, which is a notable result for a 3-step synthesis with tritiated compounds. The purified radioactive maleimides can be used directly from the preparative HPLC fractions without a solvent exchange for subsequent bioconjugations. Consequently, this allows quantitative use without loss of radioactivity and enables safe handling. The reactivity of the novel tritium-labeled maleimide derivatives could be proven by a conjugation step on thiol-functionalized oligonucleotides.

This concept opens up new possibilities for safe, fast and cost-saving access to tritium-labeled maleimide derivatives. These radiotracers find wide applications in biology and biotechnology, such as tracking biomolecules in *in vitro* and *in vivo* studies.

Experimental

Material and Methods

All chemical starting materials are commercially available and have been used without further purification. Tritium labeled [^{3}H]methyl nosylate (specific molar activity: 3.3 TBq/mmol = 88 Ci/mmol) was obtained from RC Tritec (Teufen, Switzerland) as solution in toluene. Liquid scintillation counting was accomplished using a HIDEX 300 SL and ULTIMATE GOLD™ cocktail (PerkinElmer Inc., Waltham, MA, USA). Analytical HPLC for maleimide-based derivatives were performed using an Agilent 1200 series HPLC system (Santa Clara, CA, USA) using a Waters XBridge C18 column (4.6 mm x 150 mm, 3.5 µm). HPLC conditions: mobile phase [A]: (H_2O+0.05% TFA), [B]: (acetonitrile/H_2O+0.05% TFA), gradient 10% [B] to 70% [B] over 12 min. Radiochemical purity was measured using the β Radioactivity HPLC detector RAMONA with internal solid scintillator (Elysia-raytest, Straubenhardt, Germany). Preparative purification for tritium labeled maleimides was performed by the use of Gilson PLC 2050 (Middleton, WI, USA), equipped with a Waters XBridge C18 column (10 mm x 300 mm, 5 µm) under the following conditions: Solvent [A] was water + 5% acetonitrile + 0.05% trifluoroacetic acid (*v*/*v*/*v*) and solvent [B] was acetonitrile + 0.05% trifluoroacetic acid (*v*/*v*). The column was initially equilibrated at 10% [B] using a flow rate of 6 mL/min, with the absorbance monitored at 214 nm. Starting with isocratic conditions of 10% [B] for 4 minutes, a linear gradient to 70% [B] followed over 15 minutes. The desired products eluted from the column at about 25% [B].

For purification of DA adducts, an Isco Combiflash® Rf+. (Lincoln, NE, USA) in combination with Disposable RediSep™ silica gel columns (4 g) was used for flash column chromatography under the following conditions: Solvent [A] was heptane and solvent [B] was methyl *t*-butyl ether. The column was initially equilibrated at 20% [B] using a flow rate of 18 mL/min, with the absorbance monitored at 214 nm. The elution gradient consisted of isocratic conditions at 20% [B] for 4 minutes, followed by linear gradients to 100% [B] in 14 minutes, and finally isocratic conditions at 100% [B] over 5 minutes. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance III (600 MHz) instrument. ^{1}H chemical shifts (δ) are reported in parts per million (ppm) and referenced using residual solvent resonance relative to tetramethylsilane. Signal multiplicity is described using the following abbreviations: s (singlet), d (doublet), m (multiplet), dd (doublet of doublets), and br s (broad singlet). Coupling constants (*J*) are in hertz (Hz). Tritium labeled intermediates and products were identified by HPLC comparison with commercially available non-radioactive materials or characterized compounds from Roche pharma Research and Early Development (pRED). Both oligonucleotides used as starting materials were synthesized from Roche pRED using standard phosphoramidite chemistry and consist of a complete phosphorothioate backbone. PBS buffer was purchased from Thermo Fisher Scientific (Paisley, UK), in one (1x) and tenfold (10x) concentration. Conjugated oligonucleotides were analyzed using UPLC Agilent 1290 at 260 nm wavelength and ACQUITY UPLC Oligonucleotide BEH C18, 2.1 x 50 mm, 1.7 µm column at 80 °C under the following conditions: Solvent [A] was water/methanol/hexafluoro *i*-propanol/triethylamine : 950/25/21/2.3 mL; solvent [B] was water/methanol/hexafluoro *i*-propanol/triethylamine : 175/800/21/2.3 mL, and the following linear gradient of 10% [B] to 25% [B] in 13 min by a flow of 0.5 mL/min. Concentration of conjugated oligonucleotides were determined by Eppendorf BioSprectrometer® basic (Hamburg, Germany) at 260 nm wavelength and the corresponding calculated molar extinction coefficient.

General Procedure of Maleimide Protection

To a solution of commercially available maleimide derivatives **4**, **5**, or **6** (1.42 mmol) in acetonitrile (2.0 mL) was added at 22 °C 2,5-dimethylfuran (720 mg, 800 µL, 7.5 mmol). The mixture was stirred at 65 °C in a sealed glass tube for 20 h. Removal of the solvent by evaporation and drying under high vacuum (3x 10^{-2} mbar) gave the crude Diels-Alder adduct as a light yellow oil. The *endo*/*exo* mixture was purified by flash chromatography to isolate the *exo* compound **7**, **8**, or **9** in purities of > 95% and yields in the range 55 - 60%.

General Procedure of Methylation

1.67 GBq (45 mCi) of [^{3}H]-methyl nosylate **1** (125 µg, 0.561 µmol) as solution in toluene was diluted with non-radioactive methyl nosylate (122 µg, 0.561 µmol) in a 1:1 ratio to achieve a specific molar activity of approximately 44 Ci/mmol. The solution was evaporated, transferred into a sealed tube and concentrated to dryness under an argon flow. To the solid residue was added at 22 °C a solution of DA cycloadduct **7**, **8**, or **9** (2.81 µmol) in 80 µL toluene followed by the addition of 2 M sodium *t*-butoxide solution in THF (1.7 µL, 3.37 µmol). The mixture was stirred in a sealed tube at 22 °C for 2.5 h. HPLC analysis showed the desired intermediate products **10**, **11**, or **12** with a radiochemical purity of 50%. The reaction mixture was diluted with dichloromethane (DCM) (1 mL) and directly purified by filtration through a SCX-2/SAX cartridge (Silycycle, 500 mg, pre-conditioned with DCM) to remove basic and acidic impurities. The cartridge was washed with DCM (5 mL) and the resulting solution was concentrated by evaporation to a volume of 100 µL to give the radiolabeled intermediates **10**, **11**, or **12** in radiochemical purities of > 80%. The intermediates were used without further purification for the subsequent deprotection.

General Procedure of Deprotection

The obtained crude solution of **10**, **11**, or **12** was transferred into a sealed tube, diluted with toluene (70 µL) and heated at 110 °C for 2 h.

HPLC analysis showed full conversion to the deprotected final product. The reaction mixture was allowed to cool to 22 °C. The solvent was concentrated to dryness under an argon flow. The residue was purified by preparative HPLC. The corresponding preparative HPLC fractions, containing the tritium labeled maleimide derivatives **13**, **14**, or **15**, were directly used for subsequent conjugation experiments. Radiochemical yields: 15 - 20%. Radiochemical purity: > 98%. The low sample concentration of tritiated maleimide derivatives made it impossible to determine the specific activity by mass spectrometry, since the ionization signal was too low. Due to previous dilution of hot and cold methyl nosylate in 1:1 ratio, a specific molar activity of 44 Ci/mmol is expected. Conjugation products of [^{3}H] maleimide derivatives on thiol-functionalized oligonucleotides, however, provided a clearer determination of the specific activity.

According to the *General Procedure of Maleimide Protection,* the following intermediates were synthesized:

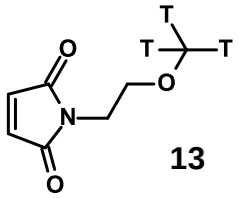
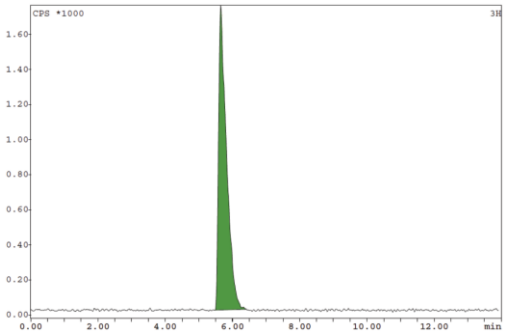
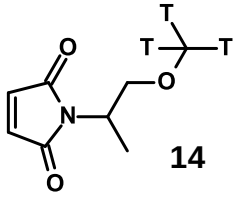
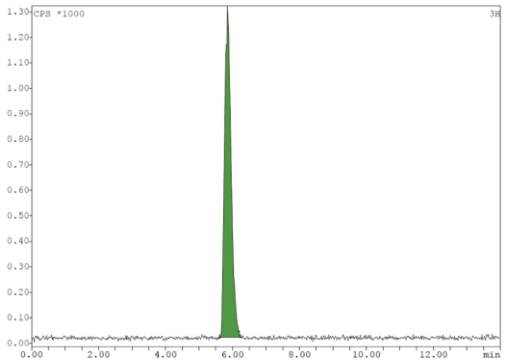
exo-2-(2-hydroxyethyl)-4,7-dimethyl-3a,7a-dihydro-4,7-epoxyisoindole-1,3-dione (**7**). This compound was isolated as a colorless solid in 55% yield and purity of > 95%; ^{1}H NMR (DMSO-

d_6) δ ppm 6.36 (s, 2 H), 4.69 (br s, 2 H), 3.41 (s, 4 H), 2.88 (s, 2 H), 1.53 (s, 6 H); ESI-MS: *m*/*z* $[M + H]^+$ calcd for $C_{12}H_{15}NO_4$: 237.1001; found: 237.1001.

exo-2-(2-hydroxy-1-methyl-ethyl)-4,7-dimethyl-3a,7a-dihydro-4,7-epoxyisoindole-1,3-dione (**8**). This compound was isolated as a colorless oil in 60% yield and purity of > 90%; ^{1}H NMR (DMSO-d_6) δ ppm 6.36 (d, *J*=1.6 Hz, 2H), 4.54 - 5.09 (m, 1 H), 3.90 – 4.15 (m, 1 H), 3.67 (dd, *J*=10.8, 8.1 Hz, 1 H), 3.49 (dd, *J*=10.8, 6.4 Hz, 1 H), 2.82 - 2.87 (m, 1 H), 2.77 - 2.81 (m, 1 H), 1.53 (d, *J*=4.0 Hz, 6 H), 1.17 (d, *J*=7.0 Hz, 3 H); ESI-MS: *m*/*z* $[M + H]^+$ calcd for $C_{13}H_{17}NO_4$: 251.1158; found: 251.1152.

exo-2-[1-(hydroxymethyl)cyclopropyl]-4,7-dimethyl-3a,7a-dihydro-4,7-epoxyisoindole-1,3-dione (**9**). This compound was isolated as a colorless solid in 60% yield and purity of > 95%; ^{1}H NMR (DMSO-d_6) δ ppm 6.35 (s, 2 H), 4.70 (br s, 1 H), 3.38 (s, 2 H), 2.78 (s, 2 H), 1.50 (s, 6 H), 0.89 – 0.93 (m, 2 H), 0.63 – 0.67 (m, 2 H); ESI-MS: *m*/*z* $[M + H]^+$ calcd for $C_{14}H_{17}NO_4$: 263.1158; found: 263.1169.

According to the *General Procedure of Deprotection*, the following products were synthesized:

Compound	*Yield [%]*	*Purity [%]*	*Radio-HPLC Trace*
 **13** *N-(2-[$^3H_3$]methoxyethyl) maleimide*	15.3	> 99	
 **14** *N-(2-[$^3H_3$]methoxy-1-methylethyl)maleimide*	13.2	> 99	

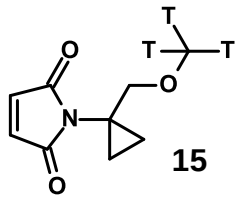
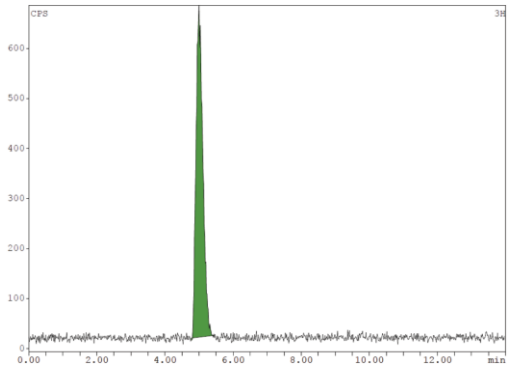
 **15** *N-[1-([$^3H_3$]methoxy-methyl)cyclopropyl] maleimide*	15.2	> 99	

Table 1: Chemical structure of **13**,**14**,**15**, overall radiochemical yield, radiochemical purity, and the corresponding radio-HPLC traces.

General Procedure of Maleimide Conjugation to Oligonucleotides

2 equivalents of oligonucleotide, containing 5'-end or 3'-end thiol-linker, was dissolved in PBS (10x) (volume factor: 250 mL/g). 1 equivalent of **13**, **14**, or **15**, directly used from preparative HPLC fraction eluent with a radio-concentration of 0.82 - 0.94 mCi/mL, was added to the aqueous oligonucleotide solution and stirred at 22 °C for 1.5 h. UPLC analysis showed a conjugation rate of 30% to 50%. 10 equivalent of the corresponding cold (non-radioactive) maleimide derivative, dissolved in THF (volume factor: 700 mL/g), was added and stirred at 22 °C for 1 h. UPLC showed a complete conjugation. The reaction mixture was transferred into an Amicon® Pro purification system (MWCO: 3,000 Da) and centrifuged at 4,000 rpm. PBS (1x) was added, the process was repeated 4 times to complete the solvent exchange and to receive the purified product. The content concentration and activity concentration of the resulting buffered solution were determined. Radiochemical yields: 69% - 99%. Specific molar activities: 10.5 Ci/mmol - 20.8 Ci/mmol. Radiochemical purities: 93.4% - 98.4%.

According to the *General Procedure of Maleimide Conjugation to Oligonucleotides*, the following oligonucleotides have been conjugated with **13**, **14**, or **15** (**Table 2**):

Sequence 5′ → 3′	*Linker*	*Label*	*Yield [%]*	*Purity [%]*	*SA [Ci/mmol]*
5'-GAGttacttgccaACT-3'	-3'-C_6-SH	**13**	69	96.0	16.5
5'-TTAcActtaattatactTCC-3'	HS-C_6-5'-	**13**	72	98.4	18.3
5'-GAGttacttgccaACT-3'	-3'-C_6-SH	**14**	87	94.3	12.0
5'-TTAcActtaattatactTCC-3'	HS-C_6-5'-	**14**	89	93.4	10.5
5'-GAGttacttgccaACT-3'	-3'-C_6-SH	**15**	90	98.1	17.0
5'-TTAcActtaattatactTCC-3'	HS-C_6-5'-	**15**	99	97.3	20.8

Table 2: Oligonucleotide-maleimide conjugates used for reactivity testing. Capital letters in sequence describe LNA modified nucleosides, small letters describe DNA nucleosides. Linker: -3'-C_6-SH = hexylene thiol at 3'-end; HS-C_6-5'- = hexylene thiol at 5'-end; SA: specific molar activity.

Conflict of interest

All authors are current employees of Roche. Roche has submitted a patent application (WO **2020**/254548 A1) covering the synthesis process of tritium *O*-methylated *N*-alkylhydroxy maleimide derivatives.

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