SYNTHESES OF [2-^{2}H]-5-ETHYNYL-1-(β-D-RIBOFURANOSYL) IMIDAZOLE-4-CARBOXAMIDE AND 5-ETHYNYL-1-([5-^{3}H]-β-D-RIBOFURANOSYL)IMIDAZOLE-4-CARBOXAMIDE (EICAR)

Noriaki Minakawa[a], Akira Matsuda[a,*],

Zuping Xia[b], Leonard I. Wiebe[b] and Edward E. Knaus[b]

[a]Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan and [b]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8

SUMMARY

Metallation of 5-ethynyl-1-(2,3,5-tri-*O-tert*-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxamide (1) using n-BuLi, deuteration with deuterium oxide and removal of the *tert*-butyldimethylsilyl protecting groups using tetrabutylammonium fluroide yielded [2-^{2}H]-5-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (5a, 75 atom % deuterium). Regiospecific deprotection of the ***masked aldehyde N,N'-diphenylethylenediamino synthon*** 14 using DIAION PK212 ion-exchange resin (H^+ form) yielded the aldehyde derivative (15). Reduction of the aldehyde moiety of 15 using excess [^{3}H]$NaBH_4$ gave the carbinol product 17. Removal of the ribofuranosyl 2,3-isopropylidene protecting group from 17 using 90% trifluoroacetic acid afforded 5-ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)imidazole-4-carboxamide (18, 19% chemical yield, > 99% radiochemical purity, specific activity 1.56 Ci/mmol).

Key words: Deuteration, [2-^{2}H]-5-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide, [^{3}H]sodium borohydride reduction, 5-ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)-imidazole-4-carboxamide, EICAR.

INTRODUCTION

EICAR [5-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide, 3] has been shown to exhibit *in vitro* cytostatic activity against various tumor cell lines including human solid

*Author to whom correspondence should be addressed.

CCC 0362-4803/96/090809-16

Received 30 January 1996
Revised 10 April 1996

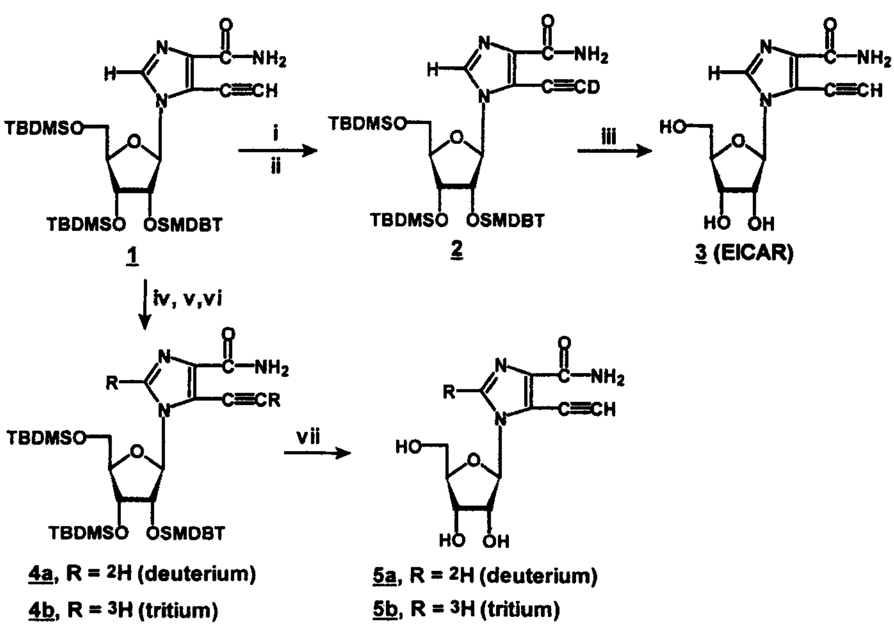
tumor cells and *in vivo* antitumor activity against murine leukemias such as L1210 and P388 (1-4). In addition to its cytostatic activity, EICAR also exhibits an antiviral activity spectrum that is similar to ribavirin (5-7). Inhibition of inosine 5'-monophosphate (IMP) dehydrogenase, which catalyzes the oxidative conversion of IMP to xanthosine 5-monophosphate (XMP) and is one of the most prominent rate-controlling enzymes of *de novo* guanine biosynthesis in mammalian systems, is believed to be responsible for the cytostatic and antiviral activities observed (8,9). Recent studies indicated that EICAR 5'-monophosphate irreversibly inactivated both human type II and *E. coli* IMP dehydrogenase (10). In the course of ongoing metabolic and mechanistic studies, it became necessary to prepare [^{2}H]- and [^{3}H]-labelled EICAR. We now report facile procedures for the syntheses of [2-^{2}H]-5-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (**5a**) and high specific activity 5-ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)imidazole-4-carboxamide (**18**).

RESULTS AND DISCUSSION

In selecting a synthetic procedure for the synthesis of [^{2}H]-labelled EICAR, it was expected that metallation of 5-ethynyl-1-(2,3,5-tri-*O-tert*-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxamide (**1**) at the terminal position of the 5-ethynyl moiety using lithium diisopropylamide (LDA), deuteration with CD_3OD and removal of the TBDMS protecting groups using tetra-n-butylammonium fluoride (TBAF) would yield the target, 5-[2-^{2}H]-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (see Scheme 1). Accordingly, reaction of **1** with two equivalents of LDA at -70 °C, followed by quenching with CD_3OD, yielded the 5-[2-^{2}H]-ethynyl product **2** in 81% chemical yield as a colorless oil. A comparison of the integrals for the imidazole C-2H at δ 7.92 and the acetylene C≡C*H* at δ 3.76 in the ^{1}H nmr spectrum of **2** indicated that the extent of deuterium incorporation on the ethynyl moiety (C≡C*D*) was about 85%. However, treatment of **2** with TBAF, to remove the TBDMS protecting groups, resulted in a complete exchange of the labile deuterium atom by a proton to afford unlabeled EICAR (**3**).

An alternative approach to incorporate either a deuterium or a tritium substituent at the C-2 position of the imidazole ring, which would be chemically and metabolically stable, was investigated (see Scheme 1). Thus, treatment of 1 with five equivalents of n-BuLi in THF at -70 °C and quenching the reaction with D_2O yielded the *bis*-deuterated product 4a in 75% chemical yield as a colorless oil. Comparison of the integrals for the imidazole C-2H and the ethynyl proton (C≡C*H*) in the ^{1}H nmr spectrum of 4a, with the correspondiong protons in the ^{1}H nmr spectrum of 1, indicated that the extent of deuterium incorporatation at the imidazole C-2 position and on the 5-ethynyl substituent were 75% and 50%, respectively. Desilylation of 4a using TBAF afforded [2-^{2}H]-5-ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (5a) as a white solid in 56% chemical yield. The extent of deuterium incorporation at the C-2

Scheme 1



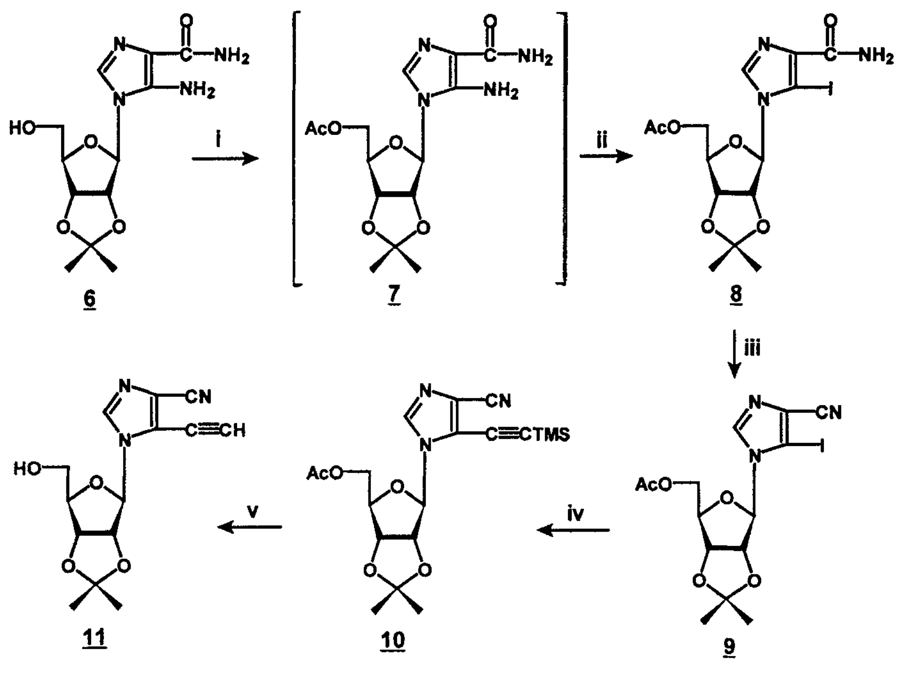
Reagents: i, LDA, THF, -70 °C; ii, CD_3OD, -70 °C; iii, TBAF, THF, 25 °C, 30 min; iv, n-BuLi, THF, -70 °C, 1 h; v, [^{2}H]H_2O (4a) or [^{3}H]H_2O (4b), -70 °C, 1h; vi, AcOH (-70 → 25 °C); vii, TBAF, 25 °C, 30 min.

position of **5a** was estimated by ^{1}H nmr spectrometry to be 75% which indicates that the C-2D subsituent was stable during the desilylation reaction using TBAF. It was expected that a similar reaction using [^{3}H]H_2O, in the place of [^{2}H]H_2O, would yield [2-^{3}H]EICAR (**5b**) having a theroretical specific activity of at least 70 mCi/mmol. However, reaction of **1** (21.7 mg, 0.0356 mmol) with 6.4 molar equivalents of *n*-BuLi (0.229 mmol), quenching with [^{3}H]H_2O (100 μL, specific activity 28 Ci/mL) and deblocking using TBAF afforded [2-^{3}H]EICAR (**5b**, 39% chemical yield, 12.4 μCi total radioactivity, specific activity 0.87 mCi/mmol, > 99% radiochemical purity). The low radiochemical yield and low specific activity of 2-[^{3}H]EICAR is most likely due to a significant isotope effect resulting from competition between the reaction of the imidazole C-2Li species with [^{1}H]H_2O relative to [^{3}H]H_2O. This explanation is in agreement with the fact that larger mass isotopes generally react slower than lower mass isotopes. Consequently, this synthetic method is not suitable for the synthesis of [2-^{3}H]EICAR (**5b**) since very high specific activity [^{3}H]H_2O is not commercially available.

An alternative synthetic strategy (see Scheme 3) was therefore investigated to circumvent the limitation encountered in the reaction using [^{3}H]H_2O for the synthesis of [2-^{3}H]EICAR **5b** (as illustrated in Scheme 1). The C-5 position of the β-D-ribofuranosyl ring system (**18**) was selected for incorporation of the tritium label since this is expected to be a metabolically stable position (see Scheme 3). It was anticipated that the target [^{3}H]-labelled EICAR (**18**) could be prepared readily from the aldehyde **15** by reduction with high specific activity [^{3}H]$NaBH_4$. Although the aldehyde **15** should be accessible by oxidation of the hydroxymethyl substituent present in **16**, it was expected that simultaneous dehydration of the 4-carbamoyl moiety to a 4-cyano substituent would also occur. Therefore, the 4-cyano derivative **11** was selected as the starting material, which was readily prepared from the known 4-carboxamido-5-amino derivative **6** (11). Compound **6** was elaborated to the required starting reagent **11** using previously reported methods (1,2), as illustrated in Scheme

2. Moffatt oxidation (12) of the hydroxymethyl moiety of 11 using 1,3-dicyclohexylcarbodiimide in DMSO (DCC/DMSO) gave aldehyde 12, which was presumed to be unstable, and so was converted to the ***masked*** aldehyde *N,N'*-diphenylethylenediamino derivative 13 in 67% yield from 11. Hydrolysis of the 4-cyano group of 13 afforded thecorresponding carboxamide derivative 14 in 78% yield. Removal of the *N,N'*-diphenylethylenediamino protecting group present in 14 using a cation exchange resin (H^+ form) in aqueous THF yielded the aldehyde 15, which without further purification, was reduced using $NaBH_4$ to afford 5-ethynyl-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxamide (16) in 59% overall yield. Removal of the 2',3'-*O*-isopropylidene protecting group of 16 by treatment with 90% trifluoroacetic acid (TFA) yielded unlabeled EICAR (3).

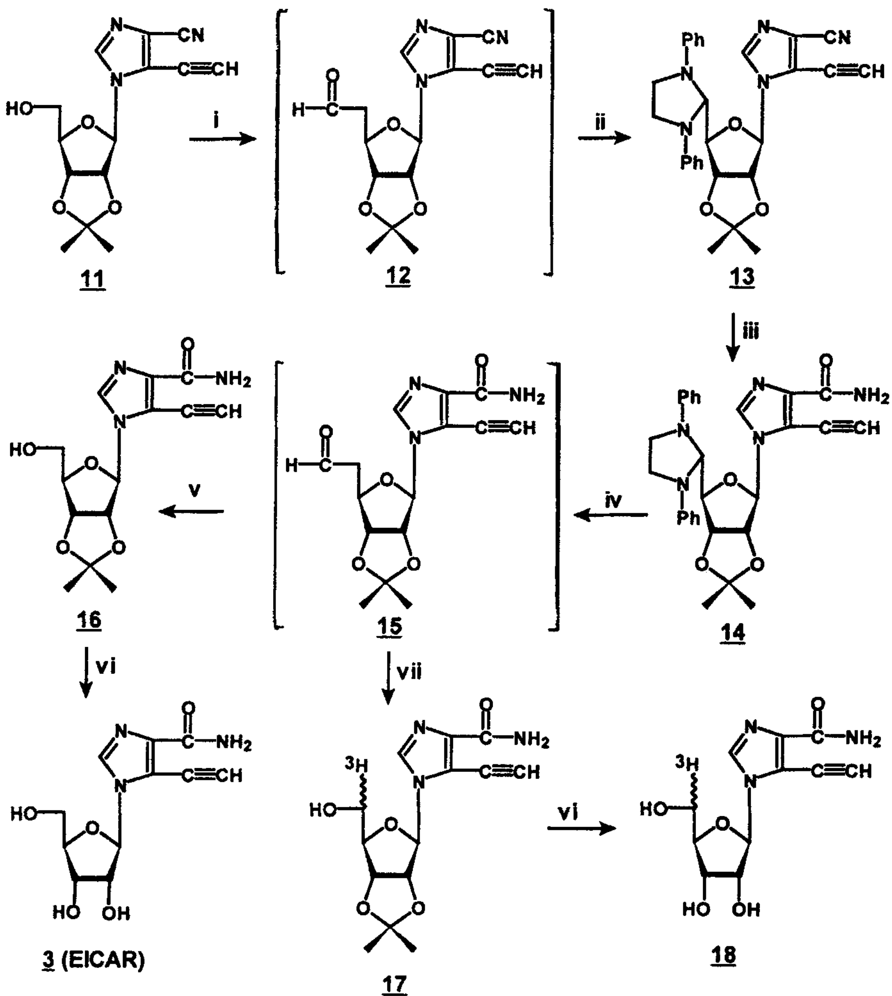
Scheme 2



Reagents: i, Ac_2O, DMAP, Et_3N, MeCN, 25 °C, 20 min; ii, CH_2I_2, isoamyl nitrite, 100 °C, 20 min; iii, $POCl_3$, Et_3N, CH_2Cl_2, 0 °C, 1 h; iv, bis(benzonitrile)palladium dichloride, TMS-C≡C-Sn-n-Bu_3, MeCN, 100 °C, 10 h; v, NH_3-MeOH, 25 °C, 2 h.

The methodology developed for the synthesis of unlabeled EICAR (3) (see Scheme 3) was used for the synthesis of [^{3}H-]-labeled EICAR (18). Thus, regiospecific deprotection of the ***masked aldehyde synthon*** (14) using DIAION PK212 ion-exchange resin (H^+ form) suspended in aqueous THF at 25 °C and a reaction time of 30 minutes yielded the aldehyde

Scheme 3



Reagents: i, DCC, DMSO, Cl_2CHCO_2H, oxalic acid; ii, *N,N'*-diphenylethylenediamine, 25 °C, 40 min; iii, NH_4OH-MeOH, H_2O_2, 25 °C, 7 h; iv, DIAION PK212 (H^+ form), THF, H_2O, 25 °C, 30 min; v, $NaBH_4$, MeOH, 25 °C, 20 min; vi, 90% CF_3CO_2H, 25 °C, 10 min; vii, [^{3}H]$NaBH_4$, MeOH, 25 °C, 30 min.

derivative (15). Reduction of 15 using excess [^{3}H]NaBH$_4$ (0.5 molar equivalents, specific activity 8.3 Ci/mmol) gave the tritiated carbinol (17) which was then treated with 90% TFA to remove the 2',3'-*O*-isopropylidene protecting group to afford 5-ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)imidazole-4-carboxamide (18, 19% chemical yield, 7 mCi total radioactivity, specific activity 1.56 Ci/mmol, > 99% radiochemical purity). A two-fold excess of [^{3}H]NaBH$_4$ (assuming four tritide anions are available for reduction of the aldehyde group) was utilized to simplify the radiosynthesis procedure. This allowed one to weigh more accurately a larger quantity of [^{3}H]NaBH$_4$, use a smaller quantity of the ***masked aldehyde synthon*** (14), and to ensure that the reduction reaction proceeded in high yield. Despite a modest 19% chemical yield of the target product 18, the high specific activity achieved (1.56 Ci/mmol) approached the theoretical maximum of 2 Ci/mmol).

SUMMARY

[2-^{2}H]-5-Ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide {[2-^{2}H]EICAR, 5a, 75 atom % deuterium} was synthesized in 42% overall chemical yield from 5-ethynyl-1-(2,3,5-tri-*O*-tert-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxamide (1) using a two step reaction sequence. High specific activity (1.56 Ci/mmol) 5-ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)imidazole-4-carboxamide (EICAR, 18) was synthesized in 14% radiochemical yield from the ***masked aldehyde synthon*** 14 using a facile three step reaction sequence.

EXPERIMENTAL

All reagents used were analytical grade. Anhydrous tetrahydrofuran (THF) was dried by distillation from calcium hydride just prior to use. Qualitative thin layer chromatography (TLC) was performed using Whatman MK6F microslides (0.25 mm thickness). Preparative TLC separations were effected using Whatman TLC plates (20 x 20 cm, 1 mm thickness). High pressure liquid chromatography (HPLC) was performed using a Waters HPLC system comprised of two Model 501 solvent pumps, Model 860 gradient flow controller, Model

U6K injector and Model 486 variable wavelength ultraviolet detector set at 263 nm using a Waters μBondapak C18 column (3.9 mm x 300 mm, P/N 27324) with MeOH-H_2O (1:1, v/v) as eluent at a flow rate of 0.5 mL/min. The identity of [^{3}H]-labelled EICAR (5b, 18) was confirmed by comparison of their reverse phase HPLC retention time with an unlabeled authentic reference standard. Radioactivity was determined by liquid scintillation counting using 10 mL OptiPhase 'HiSafe' fluor with a Beckman LS9000 liquid scintillation counter. Ultraviolet spectra (UV) and quantitative UV determinations were performed using a Philips Model 8740 UV spectrophotometer in EtOH-H_2O (1:49, v/v). [^{3}H]H_2O (specific activity 28 Ci/mL) and [^{3}H]$NaBH_4$ (specific activity 8.3 Ci/mmol) were obtained from Amersham. 5-Ethynyl-1-(2,3,5-tri-*O-tert*-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxamide (1) was prepared according to the literature procedure (13).

[2-^{2}H]-5-Ethynyl-1-(2,3,5-tri-*O-tert*-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxamide (4a).

A solution of 1 (13) (305 mg, 0.5 mmol) in dry THF (15 mL) was placed in a two-necked flask equipped with a gas inlet adaptor, thermometer, and rubber septum. To this, a solution of n-BuLi in hexane (1.5 mL of 1.62 M, 2.5 mmol) was added at a rate such that the reaction temperature did not exceed -70 °C. After the mixture was stirred for 1 hour at below -70 °C, D_2O (1 mL) was added and the whole was stirred for a further 1 hour. The reaction was then quenched by adding AcOH (1 ml) and the temperature was allowed to rise to 25 °C. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOAc (50 mL). The solution was washed successively with H_2O (15 mL), saturated aqueous $NaHCO_3$ (15 mL) and saturated brine (15 mL). The separated organic layer was dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was purified on a silica gel column (2.6 x 11 cm), eluted with 25-50% EtOAc in hexane, to give deuterated 4a (230 mg, 75% as a colorless oil).

Integration of the ^{1}H nmr spectrum of **4a** indicated that the extent of deuterium incorporation at the C-2 position of the imidazole ring and on the 5-ethynyl moiety was about 75 and 50 atom % deuterium, respectively.

[2-^{2}H]-5-Ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide {[2-^{2}H]EICAR, 5a}.

A THF solution of TBAF (1.44 mL of 1 M, 1.44 mmol) was added to a solution of **4a** (220 mg, 0.36 mmol) in THF (5 mL). The mixture was stirred for 30 min at 25 °C, and concentrated *in vacuo*. The residue was dissolved in H_2O (20 mL), which was washed with ether (3 x 20 mL). The aqueous layer was concentrated to dryness *in vacuo* and the residue was purified on a silica gel column (2.6 x 10 cm), eluted with 5-30% EtOH in $CHCl_3$, to give **5a** (54 mg, 56% as a white solid).

Integration of the ^{1}H nmr spectrum of **5a** indicated that there was about 75 atom % deuterium at the C-2 position of the imidazole ring.

[2-^{3}H]-5-Ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (5b).

n-Butyllithium (0.143 mL of a 1.6 M solution in hexane, 0.229 mmol, 6.4 equivalents) was added to a solution of **1** (21.7 mg, 0.0356 mmol) in dry THF (1 mL) precooled to -65 °C under an argon atmosphere with stirring. The reaction mixture was maintained at -65 °C for 1 hour, [^{3}H]H_2O (0.1 mL, specific acitivity 28 Ci/mL) was added, the reaction mixture was stirred for 1 hour at -65 °C, AcOH (50 μL) was added to quench the reaction and the stirred reaction mixture was allowed to warm to 25 °C. This solution was applied onto a Waters C-18 sep-pak cartridge column that was eluted with H_2O (3 mL) and then EtOAc (6 mL). A small aliquot (about 0.1 μL) of each fraction collected (0.4 mL) was applied to a Whatman MK6F silica gel microslide that was not developed. The solvent, from those fractions which exhibited a UV visible spot (254 nm) on the silica gel microslide above, was removed *in vacuo* to afford **4b** as a solid which was used immediately in the subsequent reaction without further purification. A solution of **4b**, obtained above, in THF (0.5 mL) and a solution of TBAF (1M in THF, 142 μL) was stirred for 30 minutes at 25 °C, the solvent was removed *in vacuo* and the residue obtained was dissolved in H_2O (0.5 mL). An aqueous solution of 1 M

$NaClO_4$ (152 μL) was added and the resulting precipitate was removed by filtration. The filtrate obtained was washed with ether (2 x 3 mL), the ether wash was discarded, and the volume of the aqueous fraction was reduced until a precipitate formed that was removed by filtration and discarded. The aqueous filtrate was then applied onto a Waters C-18 sep-pak cartridge column which was eluted with H_2O (15 mL). A small aliquot (about 0.1 μL) of each fraction collected (0.4 mL) was applied to a Whatman MK6F silica gel microslide that was not developed. The solvent, from those fractions which exhibited a UV visible spot (254 nm) on the silica gel microslide above, was removed *in vacuo* to afford **5b** which was formulated as a solution in EtOH-H_2O (1:49, v/v, 1.4 mL). The radiochemical purity of this solution of **5b** was determined by HPLC analysis and liquid scintillation counting (LSC). All of the radioactivity was associated with the eluting peak collected at 5.9 minutes (12.4 μCi total radioactivity, > 99% radiochemical purity), which was identical to the retention time of an authentic sample of unlabeled EICAR (**3**). The UV spectrum exhibited by the solution of [^{3}H]-**5b** was also identical to that for an unlabeled authentic sample of **3**. Quantitative UV analysis of the solution of [^{3}H]-**5b** described above indicated that the chemical yield was 39% (3.7 mg) and that the specific activity was 0.87 mCi/mmol.

5-Iodo-1-(5-*O*-acetyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxamide (8).

Triethylamine (8.3 mL, 60 mmol) was added to a solution of **6** (11) (11.92 g, 40 mmol) in dry acetonitrile (100 mL) containing acetic anhydride (5.6 mL, 60 mmol) and DMAP (50 mg). The reaction mixture was stirred for 20 minutes at 25 °C and EtOH (10 mL) was added to the mixture to decompose the excess acetic anhydride. The mixture was concentrated to dryness *in vacuo* and the residue was dissolved in $CHCl_3$ (400 mL), which was washed with saturated aqueous $NaHCO_3$ (2 x 100 mL), followed by saturated brine (100 ml). The separated organic layer was dried (Na_2SO_4) and concentrated to dryness to give crude **7**. A CH_2Cl_2 solution of **7** (40 mL) was added dropwise to a solution of diiodomethane (150 mL) containing isoamyl nitrite (15 mL, 112 mmol) at 100 °C. After being stirred for 20 minutes, the reaction mixture was absorbed onto a silica gel column (5.8 x 27 cm), which was washed with $CHCl_3$ to remove diiodomethane, and then eluted with 1-4 % EtOH in $CHCl_3$, to give **8** (10.39 g, 58% as a yellow foam): MS *m/z* 451 (M^+); ^1H nmr ($CDCl_3$) δ 7.86 (s, 1H, H-2), 7.04 (br s, 1H, amide proton), 5.98 (d, 1H, H-1', $J_{1',2'}$ = 2.8 Hz), 5.48 (br s, 1H, amide proton), 4.86 (dd, 1H, H-2', $J_{1',2'}$ = 2.8, $J_{2',3'}$ = 6.3 Hz), 4.79 (dd, 1H, H-3', $J_{2',3'}$ = 6.3, $J_{3',4'}$ = 3.8 Hz), 4.47 (m, 1H, H-4'), 4.34 (m, 2H, H-5'a, b), 2.10 (s, 3H, $COCH_3$), 1.38 and 1.64 (two s, 3H each, CMe_2).

5-Iodo-1-(5-*O*-acetyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carbonitrile (9).

Phosphorus oxychloride (0.56 mL, 6.0 mmol) was added dropwise to a solution of **8** (1.8 g, 4.0 mmol) in dry CH_2Cl_2 (30 mL) containing Et_3N (2.79 mL, 20 mmol) at 0 °C and the whole was stirred for 1 hour at 0 °C. The reaction was quenched by addition of crushed ice. The solution was diluted with $CHCl_3$ (80 mL), which was washed successively with H_2O (2 x 40 ml), saturated aqueous $NaHCO_3$ (40 mL) and saturated brine (40 mL). The separated organic layer was dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was purified on a silica gel column (3.6 x 9 cm), eluted with 25-50% EtOAc in hexane, to give **9** (1.52 g, 88% as a yellow oil): MS *m/z* 433 (M^+); ^1H nmr ($CDCl_3$) δ 7.86 (s, 1H, H-2), 5.83 (d, 1H, H-1', $J_{1',2'}$ = 2.0 Hz), 4.80 (m, 2H, H-2', H-3'), 4.52 (m, 1H, H-4'), 4.34 (m, 2H, H-5'a,b), 2.08 (s, 3H, $COCH_3$), 1.34 and 1.69 (two s, 3H each, CMe_2).

5-Trimethylsilylethynyl-1-(5-*O*-acetyl-2,3-*O*-isopropylidene-β-D-ribofuranosyl) imidazole-4-carbonitrile (10).

A mixture of **9** (433 mg, 1.0 mmol), *bis*(benzonitrile)palladium dichloride (36 mg, 10 mol%), trimethyl[(tributylstannyl)ethynyl]silane (580 mg, 1.5 mmol) in dry acetonitrile (5 mL) in a sealed glass tube was heated at 100 °C for 10 hours. The reaction mixture was filtered through a Celite pad and washed with EtOH. The combined filtrate and washings were concentrated to dryness *in vacuo* and the residue was purified on a silica gel column (2.8 x 12 cm), eluted with 25-33% EtOAc in hexane, to give **10** (349 mg, 87% as a brown oil): MS *m/z*

403 (M^+); ^{1}H nmr ($CDCl_3$) δ 7.66 (s, 1H, H-2), 5.91 (d, 1H, H-1', $J_{1',2'}$ = 2.4 Hz), 4.95 (dd, 1H, H-2', $J_{1',2'}$ = 2.4, $J_{2',3'}$ = 6.3 Hz), 4.75 (dd, 1H, H-3', $J_{2',3'}$ = 6.3, $J_{3',4'}$ = 3.2 Hz), 4.55 (m, 1H, H-4'), 4.30 (m, 2H, H-5'a, b), 2.01 (s, 3H, $COCH_3$), 1.37 and 1.60 (two s, 3H each, CMe_2), 0.30 (s, 9H, $SiMe_3$).

5-Ethynyl-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carbonitrile (11).

Compound 10 (343 mg, 0.85 mmol) was dissolved in NH_3/MeOH (saturated at 0 °C, 10 mL), and the mixture was stirred for 2 hours at 25 °C. The solvent was removed *in vacuo* and the residue was purified on a silica gel column (2.6 x 8 cm), eluted with 50-75% EtOAc in hexane, to give 11 (242 mg, 98% as a brown oil): MS *m/z* 289 (M^+); ^{1}H nmr ($CDCl_3$) δ 8.06 (s, 1H, H-2), 5.93 (d, 1H, H-1', $J_{1',2'}$ = 3.3 Hz). 4.94 (m, 2H, H-2', H-3'), 4.46 (br s, 1H, H-4'), 3.91 (m, 2H, H-5'a, b), 3.89 (s, 1H, C≡C*H*), 3.46 (dd, 1H, 5'-OH, *J* = 3.8, 4.4 Hz), 1.38 and 1.60 (two s, 3H each, CMe_2).

5-Ethynyl-1-[5-deoxy-5,5-(*N,N'*-diphenylethylenediamino)-2,3-*O*-isopropylidene-β-D-ribofuranosyl]imidazole-4-carbonitrile (13).

Compound 11 (289 mg, 1.0 mmol) and DCC (618 mg, 3.0 mmol) were dissolved in dry DMSO (5 mL) and the mixture was cooled in an ice bath under an argon atomosphere. Dichloroacetic acid (41 μL, 0.5 mmol) was added to the mixture and the whole was stirred for 1.5 hours at 25 °C. To the mixture, a MeOH solution (2 mL) of oxalic acid (180 mg, 2.0 mmol) was added dropwise and stirred for 30 minutes. The reaction mixture was filtered through a Celite pad and the residue was washed with ice-cold MeOH. To the combined filtrate and washings, *N,N'*-diphenylethylenediamine (254 mg, 1.2 mmol) was added and the mixture was stirred for 40 minutes at 25 °C. The mixture was concentrated to dryness *in vacuo* and the residue was dissolved in EtOAc (100 mL), which was washed with H_2O (3 x 30 mL), followed by saturated aqueous NaCl (30 mL). The separated organic layer was dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was purified on a silica gel column (by using neutralized silica gel; ICN Silica 63-200, 60 Å, 2.3 x 8 cm), eluted with 20-33% EtOAc in hexane, to give 13 (321 mg, 67% as a white foam, recrystallized from

MeOH): mp 141-143 °C; MS *m/z* 405 (M^+ - Ph); ^{1}H nmr ($CDCl_3$) δ 7.27-7.35 (m, 5H, C_6H_5), 6.89-6.80 (m, 6H, H-2, C_6H_5), 5.80 (d, 1H, H-5', $J_{4',5'}$ = 2.8 Hz), 5.79 (s, 1H, H-1'), 4.92 (m, 1H, H-2'), 4.61 (m, 2H, H-3', H-4'), 3.70 (s, 1H, C≡C*H*), 3.68 (m, 4H, NCH_2CH_2N), 1.30 and 1.45 (two s, 3H each, C*Me*$_2$). *Anal.* Calcd. for $C_{28}H_{27}N_5O_3$: C, 69.84; H, 5.65; N, 14.54. Found: C, 69.72; H, 5.73; N, 14.42.

5-Ethynyl-1-[5-deoxy-5,5-(*N,N'*-diphenylethylenediamino)-2,3-*O*-isopropylidene-β-D-ribofuranosyl]imidazole-4-carboxamide (14).

A suspension of 13 (610 mg, 1.27 mmol) in NH_4OH-MeOH (5mL-10mL) containing H_2O_2 (30% w/v, 0.5 mL) was stirred for 7 hours at 25 °C. The mixture was concentrated to dryness *in vacuo* and the residue was dissolved in EtOAc (100 mL), which was washed with H_2O (3 x 30 mL), followed by saturated aqueous NaCl (30 mL). The separated organic layer was dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was purified on a silica gel column (by using neutralized silica gel, 2.6 x 13 cm), eluted with 50-100% EtOAc in hexane, to give 14 (491 mg, 78% as a white foam); MS *m/z* 499 (M^+); ^{1}H nmr ($CDCl_3$) δ 7.29-7.35 (m, 5H, C_6H_5), 6.81-6.88 (m, 7H, H-2, amide proton, C_6H_5), 5.89 (d, 1H, H-5', $J_{4',5'}$ = 2.7 Hz), 5.80 (s, 1H, H-1'), 5.45 (br s, 1H, amide proton), 4.92 (m, 1H, H-2'), 4.67 (m, 1H, H-3'), 4.60 (m, 1H, H-4'), 3.68 (s, 1H, C≡C*H*), 3.68 (m, 4H, NCH_2CH_2N), 1.31 and 1.49 (two s, 3H each, C*Me*$_2$).

5-Ethynyl-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxamide (16).

A solution of 14 (100 mg, 0.2 mmol) in a mixture of H_2O (2 mL) and THF (2 mL) was stirred for 30 minutes at 25 °C in the presence of DIAION PK212 (Mitsubishi Kasei Co., H^+ form, 2 mL as wet volume). The resin was filtered off and washed with hot MeOH. The combined filtrate and washings were concentrated to dryness *in vacuo*. The residue 15 was dissolved in MeOH (4 mL) and $NaBH_4$ (23 mg, 0.6 mmol) was added to the solution. The mixture was stirred for 20 minutes at 25 °C. The mixture was concentrated to dryness *in vacuo* and the residue was coevaporated three times with MeOH. The residue was purified on a silica gel column (2.1 x 5 cm), eluted with 4-16% EtOH in $CHCl_3$, to give 16 (36 mg, 59%

as a colorless glass, recrystallized from EtOH-hexane): mp 145-146 °C; MS *m/z* 307 (M^+); ^1H nmr ($CDCl_3$) δ 7.82 (s, 1H, H-2), 6.95 (br s, 1H, amide proton), 5.96 (d, 1H, H-1', $J_{1',2'}$ = 3.3 Hz), 5.64 (br s, 1H, amide proton), 5.00 (dd, 1H, H-2', $J_{1',2'}$ = 3.3, $J_{2',3'}$ = 6.6 Hz), 4.94 (dd, 1H, H-3', $J_{2',3'}$ = 6.6, $J_{3',4'}$ = 3.3 Hz), 4.34 (ddd, 1H, H-4', $J_{3',4'} = J_{4',5'a} = J_{4',5'b}$ = 3.3 Hz), 3.93 (m, 1H, H-5'a), 3.86 (s, 1H, C≡C*H*), 3.81 (m, 1H, H-5'b), 2.80 (br s, 1H, 5'-OH), 1.37 and 1.61 (two s, 3H each, C*Me*$_2$). *Anal.* Calcd. for $C_{14}H_{17}N_3O_5$: C, 54.72; H, 5.58; N, 13.67. Found: C, 54.39; H, 5.55; N, 13.56.

5-Ethynyl-1-(β-D-ribofuranosyl)imidazole-4-carboxamide (EICAR, 3).

An aqueous TFA solution (90% w/v, 2 mL) containing 16 (36 mg, 0.12 mmol) was stirred for 10 minutes at 25 C. The solvent was removed *in vacuo* and the residue was coevaporated several times with EtOH. The residue was purified on a silica gel column (1.7 x 2 cm), eluted with 5-30% EtOH in $CHCl_3$, to give 3 (25 mg, 80% as a colorless glass). The physical data for 3 has been reported previously (2).

5-Ethynyl-1-([5-^{3}H]-β-D-ribofuranosyl)imidazole-4-carboxamide (18).

Wet DIAION PK212 ion-exchange resin (H^+ form, about 0.2 mL), which was exposed to 5 N HCl (10 mL) for 12 hours and washed with water until the water wash was neutral, was added to a solution of 14 (12 mg, 0.024 mmol) in H_2O (0.2 mL) and THF (0.2 mL). This mixture was stirred for 30 minutes at 25 °C, after which the solution was transferred using a syringe, to a round bottom flask (5 mL volume). The solvent was removed *in vacuo*, the residue obtained (15) was dissolved in MeOH (0.4 mL) and [^{3}H]$NaBH_4$ (470 μg, 0.012 mmol, 99 mCi of specific activity 8.3 Ci/mmol) was added. The reaction was allowed to proceed for 30 minutes with stirring at 25 °C, and the reaction mixture was applied to a preparative silica gel plate which was developed using MeOH-$CHCl_3$ (15:85, v/v) as development solvent. The TLC spot, having R_f 0.42 which was identical to that of unlabeled 17, was removed from the silica gel plate and EtOH-$CHCl_3$ (1:4, v/v; 10 mL) was added to this silica gel fraction. Removal of the silica gel by filtration, washing the silica gel with EtOH (2 x 2 mL) and removal of the solvent *in vacuo* from the combined filtrate and washings afforded 17. Addition of 90% TFA (0.6 mL) to the residue 17, allowing the reaction to proceed for 15 minutes at 25 °C with stirring, and then removal of the solvent *in vacuo* gave a residue (18) which was dissolved in EtOH (0.5 mL). This solution was applied to one preparative silica gel TLC plate that was developed using MeOH-$CHCl_3$ (1:4, v/v). Removal of the silica gel spot having R_f 0.22, which was identical to that of an unlabeled authentic reference standard, addition of EtOH (10 mL) to the silica gel fraction, removal of the silica gel by filtration, washing the silica gel with EtOH (2 x 2 mL) and removal of the solvent *in vacuo* from the combined filtrate and washings afforded the pure product 18 which was immediately formulated for storage as a solution in EtOH-H_2O (1:1, v/v; 1.5 mL). The radiochemical purity of 18 in this solution was determined by HPLC analysis and liquid scintillation counting. All of the radioactivity was associated with the eluting peak collected at 5.9 minutes (7.0 mCi total radioactivity, > 99% radiochemical purity), which was identical to the retention time of an authentic sample of unlabeled 18. Quantitative HPLC analysis of the solution of [5-^{3}H]-18 described above indicated that the chemical yield of [5-^{3}H]-18 was 19% (1.2 mg) and that the specific acitivity was 1.56 Ci/mmol.

Acknowledgements

We are grateful to the Medical Research Council of Canada (Grant MT-12304) for financial support of this research, to Dr. John Mercer for valuable suggestions pertaining to the safe handling of radioactive materials and manipulation experimental procedures and to Amersham for a generous gift of [^{3}H]H_2O.

REFERENCES

1. Matsuda A., Minakawa N., Sasaki T. and Ueda T. — *Chem. Pharm. Bull. 36*: 2730 (1988).
2. Minakawa N., Takeda T., Sasaki T., Matsuda A. and Ueda T. — *J. Med. Chem. 34*: 778 (1991).

3. Minakawa N., Matsuda A. Ueda T. and Sasaki T. — *Nucleosides Nucleotides* ***9***: 1067 (1990).

4. Minakawa N., Kojima N., Sasaki T. and Matsuda A. — *Nucleosides Nucleotides* **15**: 251 (1996).

5. De Clercq E., Cools M., Balzarini J., Snoeck R., Andrei G., Hosoya M., Shigeta S., Ueda T., Minakawa N. and Matsuda A. — *Antimicrob. Agents Chemother.* ***35***: 679 (1991).

6. Shigeta S., Mori S., Baba M., Ito M., Hozumi K., Nakamura K., Oshitani N., Numazaki Y., Matsuda A., Obara T. and Shuto S. — *Antimicrob. Agents Chemother.* ***36***: 435 (1992).

7. Balzarini J., Lee C., Schols D. and De Clercq E. — *Biochem. Biophys. Res. Commun.* ***178***: 563 (1991).

8. Balzarini J. and De Clercq E. — *Biochem. J.* ***287***: 785 (1992).

9. Balzarini J., Karlsson A., Wang L., Bohaman C., Horska K., Votruba I., Fridland A. Van Aerschot A., Herdewijn P. and De Clercq E. — *J. Biol. Chem.* ***268***: 24591 (1993).

10. Wang W., Papov V. V., Minakawa N., Matsuda A., Biemann K. and Hedstrom L. — *Biochemistry* **35**: 95 (1996).

11. Srivastava P. C., Newman A. R., Matthews T. R. and Robins R. K. — *J. Med. Chem.* ***18***: 1237 (1975).

12. Ranganathan R. S., Jones G. H. and Moffatt J. G. — *J. Org. Chem.* ***39***: 290 (1974).

13. Minakawa N. and Matsuda A. *Tetrahedron* ***49***: 557 (1993).