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SYNTHESIS OF [^{3}H]-LABELLED GLUCOBRASSICIN, A POTENTIAL RADIOTRACER FOR METABOLIC STUDIES OF INDOLE GLUCOSINOLATES

S. Chevolleau[a], B. Joseph[b], P.Rollin[b] and J. Tulliez[a]*

a) Laboratoire des Xénobiotiques, INRA, B.P. 3, 31931 Toulouse Cedex, France
b) LCBA / URA CNRS 499, Université d'Orléans, B.P. 6759, 45067 Orléans Cedex 2, France
* Author to whom correspondence should be addressed

ABSTRACT

Glucobrassicin, an indole glucosinolate widespread in cruciferous plants, appears to be involved in anticarcinogenic activity *via* its breakdown products. In order to study the "in vivo" metabolism of glucobrassicin, we have synthesized the 5-[^{3}H]-indol-3-ylmethyl glucosinolate (^{3}H-labelled glucobrassicin) starting from artificial peracetylated 5-bromoindol-3-ylmethyl glucosinolate. The labelled compounds were characterized by radio-HPLC and FAB mass spectrometry.

KEYWORDS : Glucosinolates, Glucobrassicin, Anti-carcinogen, Tritium, Catalytic hydrogenation.

INTRODUCTION

In recent years, research reports from several laboratories have rather strongly suggested that some naturally-occurring plant products can provide protection against carcinogenesis (1). These compounds may either act protectively to lessen or eliminate the effects of carcinogenic agents or hinder the active metabolites generated from precursors.

Indol-3-yl carbinol is one of these chemopreventive agents, derived from glucobrassicin, a glucosinolate present in most cruciferous vegetables (broccoli, Brussels sprouts, cabbages, cauliflowers). Its formation takes place in moist conditions and under the action of the co-existing endogenous enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1.).

Studies of the degradation mechanism of glucobrassicin have already been effected "*in*

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vitro", using material of extractive origin (2,3,4), and the "*in vivo*" fate of indol-3-yl carbinol has only been studied using the ^{3}H labelled molecule in rainbow trout (5).

Nevertheless, its is not known to what extent this degradation product may occur before the vegetables are ingested, or appears in the intestinal tract of the animals as the result of bacterial thioglucosidase action. Therefore the study of the "*in vivo*" biotransformations of the original glucobrassicin is a critical issue. The synthesis of radiolabelled glucobrassicin is therefore mandatory to follow the fate of this compound.

The synthesis of unlabelled glucobrassicin has already been described in a previous work (6).

In this paper, we report the synthesis of the peracetylated 5-bromoindol-3-ylmethyl glucosinolate and the subsequent preparation of labelled glucobrassicin through tritiation of the former compound, according to a method proposed by Dashwood *et al* (7).

EXPERIMENTAL

GENERAL :

Palladium/C (10%) and triethylamine were purchased from Aldrich. Acetonitrile was purchased from Carlo Erba and methanol Uvasol from Merck. Potassium was purchased from Prolabo.

Pd/C elimination from the mixture was effected by filtration-centrifugation on Ultrafree-MC 0.45 µm (Millipore).

Thin layer chromatography was run on aluminium plates precoated with silica gel $60F_{254}$ (Merck); detection was effected by observation under short wavelength UV light (254 nm), then dipping the chromatograms into a solution of ceric ammonium nitrate $[Ce(NH_4)_2(NO_3)_6]$ in 20% sulfuric acid and charring them with a heat gun.

Column chromatography was performed using silica gel 60 (0,063-0,200 mm, Merck) and flash chromatography was conducted with silica gel (0,040-0,063 mm, Merck).

HPLC analyses were carried out using a Kontron Model 420 pump with a gradient former 425, equipped with a Rheodyne injector. UV detection was carried out with a Kontron Model 432 detector and radioactivity detection with a Radiomatic Flo-one A250 instrument and Luma flow II scintillation cocktail.

An ODS3 Partisil column (250mm, 4.6mm, 5µm) from SFCC-Shandon was used for the purifications and analyses. The mobile phases were A: CH_3CN/ NaH_2PO_4 10mM buffer pH3.2 10/90 (v/v) and B: CH_3CN/ NaH_2PO_4 10mM buffer pH3.2 30/70 (v/v) under isocratic or gradient elution mode, with U.V. detection at 235 nm.

Optical rotations were measured with a Jobin-Yvon Digital type 71 polarimeter at 22°C.

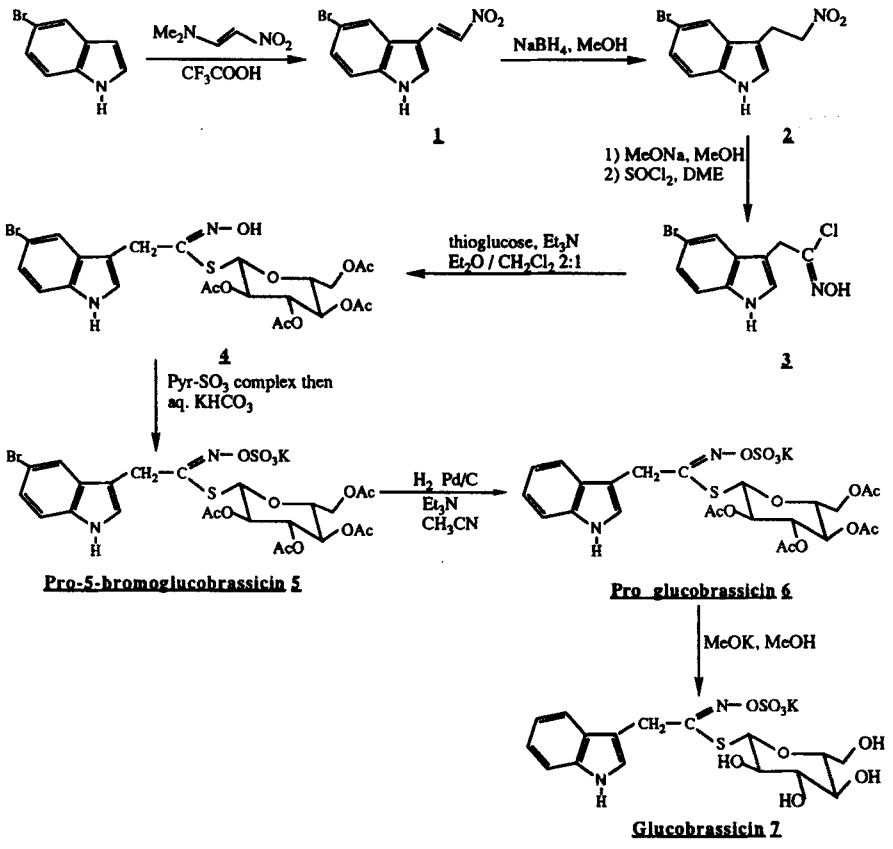
^{1}H NMR spectra were recorded at 300 K on a Bruker AM 300 (300.13 MHz for ^{1}H) spectrometer; chemical shifts are expressed in parts per million downfield from TMS.

Mass spectra were recorded (LSIMS mode) on a VG-Autospec-Q apparatus (for compounds **4**, **5**).

For compounds 6, 7, 6', 7', a Nermag R-10-10 H quadrupole mass spectrometer was used. The mass spectrometer was fitted with an M-Scan FAB gun and xenon gas was used for bombardment, operating at 8 KV accelerating voltage and 1-2 mA as discharge current. The samples were prepared by mixing 1µl of the sample solution (2mg/ ml in methanol) with 1µl of the matrix (glycerol for negative ion experiments and glycerol + KCl for positive experiments).

SYNTHESIS :

The synthesis of glucobrassicin 7 has firstly been developed on the unlabelled compounds (6).The different steps of the synthetic pathway are summarized in scheme 1.



Scheme 1 : Synthesis of glucobrassicin.

5-Bromo-3-(2-nitrovinyl)indole 1

Following the procedure of Büchi and Mak (8), 5-bromoindole (1 g, 5.1 mmol) and N,N-dimethyl-2-nitrovinylamine (9) (592 mg, 5.1 mmol) were dissolved in trifluoroacetic acid (7

ml) kept at 0°C under argon. After 1h stirring at room temperature, the mixture was poured into water, then extracted several times with ethyl acetate. The combined organic layers were washed (5% aqueous $NaHCO_3$, then brine), dried over $MgSO_4$ and concentrated. Recrystallization from methanol gave **1** (1.09 g, 80% yield) in the form of bright orange prisms, mp 197-8°C (lit. (10) 196.5-198.5°C).

^{1}H-Nmr [$(CD_3)_2SO$] δ: 7.38 (1H, *dd*, $J_{6,7}$ 8.7 Hz, $J_{6,4}$ 1.4 Hz, 6-H), 7.47 (1H, *d*, 7-H), 8.09 (1H, *d*, $J_{9,8}$ 14.0 Hz, 9-H), 8.22 (1H, *d*, 4-H), 8.27 (1H, *s*, 2-H), 8.38 (1H, *d*, 8-H), 11.20 (1H, *bs*, NH).

5-Bromo-3-(2-nitroethyl)indole **2** was prepared according to Still et al. (10); mp 93°C (lit. 91-92.5°C).

^{1}H-Nmr ($CDCl_3$) δ: 3.44 (2H, *t*, J_{vic} 7.1 Hz, 8ab-H), 4.65 (2H, *t*, 9ab-H), 7.08 (1H, *d*, $J_{2,NH}$ 2.5 Hz, 2-H), 7.26 (1H, *d*, *J7,6* 8.8 Hz, 7-H), 7.31 (1H, *dd*, $J_{6,4}$ 1.4 Hz, 6-H), 7.70 (1H, *d*, 4-H), 8.09 (1H, *bs*, NH).

5-Bromoglucobrassicin thiohydroximate 4

To a stirred solution of sodium methoxide (85 mg, 3.7 mmol of sodium in 5 ml of anhydrous methanol) was added a solution of **2** (1 g, 3.7 mmol) in a minimum volume of anhydrous methanol (5 ml). After 10 min. stirring, the solution was evaporated to give the raw nitronate, which was suspended under an argon atmosphere in cooled (-40°C) anhydrous DME (5 ml). A solution of thionyl chloride (0.3 ml, 4.1 mmol) in DME (2 ml) was then added via a syringe. After stirring at -40°C for 30 min, the mixture was treated with water (7 ml), most of the DME evaporated, followed by extraction with CH_2Cl_2. The dichloromethane extract was dried ($MgSO_4$), then evaporated under reduced pressure to give the crude (5-bromo)indol-3-ylacethydroximoyl chloride **3** which was used in the next step without further purification.

To a stirred solution of crude **3** (433 mg, 1.51 mmol) in anhydrous diethyl ether-dichloromethane 2:1 v/v (10 ml) under argon were successively added 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose (458 mg, 1.26 mmol) in dry dichloromethane (5 ml) and freshly distilled triethylamine (0.52 ml, 3.73 mmol). Triethylammonium hydrochloride immediately precipitated from the solution. After stirring for 1h, the mixture was acidified with 0.5 M H_2SO_4, extracted with dichloromethane and the organic layer was dried ($MgSO_4$) and evaporated to dryness. The remaining solid was purified by column chromatography using dichloromethane/methanol (98:2 v/v) to afford amorphous **4** (558 mg, 72% yield); $[\alpha]_D$ +3° (*c* 1.0 in $CHCl_3$).

^{1}H-Nmr ($CDCl_3$) δ: 1.96, 1.97, 2.00 and 2.07 (4x3H, 4*s*, OAc), 3.42 (1H, *m*, 5-H), 3.96 and 4.02 (2H, 2*d*, J_{gem} 16.6 Hz, 8b-H and 8a-H), 4.03 (1H, *dd*, $J_{6b,5}$ 2.6 Hz, J_{gem} 12.6 Hz, 6b-H), 4.12 (1H, *dd*, $J_{6a,5}$ 5.5 Hz, 6a-H), 4.92-5.08 (4H, *m*, 1-H, 2-H, 3-H, 4-H), 7.05 (1H, *d*, $J_{2i,NH}$ 2.2 Hz, 2i-H), 7.23 (1H, *d*, $J_{7i,6i}$ 8.7 Hz, 7i-H), 7.28 (1H, *dd*, $J_{6i,4i}$ 1.6 Hz, 6i-H), 7.71 (1H, *d*, 4i-H), 8.55 (1H, *d*, NH), 9.16 (1H, *bs*, NOH).

m/z for $C_{24}H_{27}BrN_2O_{10}S$: 637 and 639 $(M+Na)^+$.

Pro-5-bromoglucobrassicin 5

To a cooled (0°C) and stirred solution of pyridine (4 ml) in dry dichloromethane (4 ml) under argon, a solution of chlorosulphonic acid (0,18 ml, 2,63 mmol) in dry dichloromethane (4 ml) was added over a period of 15 min, followed by a solution of 4 (160 mg, 0,26 mmol) in dry dichloromethane (2 ml). After stirring for 24h at room temperature, the medium was treated with a solution of potassium hydrogen carbonate (160 mg, 1.6 mmol) in water (5 ml) and stirred another 30 min; the solvents were evaporated and traces of pyridine removed by coevaporation with toluene. The residue was purified by column chromatography (eluent methanol/dichloromethane 3:17 v/v) to give 5 (166 mg, 87% yield) as an amorphous powder; $[\alpha]_D$-3° (*c* 1.1 in MeOH).
^{1}H-Nmr [$(CD_3)_2SO$] δ: 1.90, 1.92, 1.93 and 1.97 (4x3H, 4*s*, OAc), 3.80 (1H, *bd*, J_{gem} 10.9 Hz, 6b-H), 3.93-4.08 (4H, *m*, 5-H, 6a-H, 8ab-H), 4.82 (1H, *ft*, J_{vic} 9.7 Hz, 2-H), 4.90 (1H, *ft*, J_{vic} 9.7 Hz, 4-H), 5.30 (1H, *ft*, $J_{3,2}$ 9.5 Hz, 3-H), 5.47 (1H, *d*, $J_{1,2}$ 10.0 Hz, 1-H), 7.18 (1H, *dd*, $J_{6i,7i}$ 8.7 Hz, $J_{6i,4i}$ 1.6 Hz, 6i-H), 7.32 (1H, *d*, 7i-H), 7.43 (1H, *d*, $J_{2i,NH}$ 2.4 Hz, 2i-H), 7.82 (1H, *d*, 4i-H), 11.17 (1H, *d*, NH).
m/z for $C_{24}H_{26}BrKN_2O_{13}S_2$: 733 and 735 $(M+1)^+$.

HPLC analysis gave a retention time of 15.8 min for Pro-5-bromoglucobrassicin (mobile phase B: flow rate 1ml/min).

Proglucobrassicin 6

A flask equipped with a magnetic stirrer and containing 2 mg of 5, 16 mg of Pd/C (10%), 5µl of triethylamine and 4 ml of acetonitrile was placed in an hydrogenation apparatus.The mixture was stirred under hydrogen (5 bars) for 3h.

After this period, Pd/C was removed by filtration-centrifugation and an aliquot of reaction mixture was evaporated to dryness and the residue was diluted with mobile phase B:.

HPLC analysis gave a retention time of 8.9 min (mobile phase B: flow rate 1 ml/min) for proglucobrassicin 6 (ProGBS) with a purity of 75%.

HPLC purification of 6 was carried out by several injections and collect of the peak corresponding to ProGBS. After removal of the mobile phase, Pro GBS 6 was analyzed by negative (NI) and positive (PI) ion FAB mass spectrometry.

Under NI-FAB conditions, the pseudo-molecular ion of Pro GBS is observed at m/z 615 for $C_{24}H_{27}O_{13}N_2S_2$, corresponding to the $(MK-K)^-$ form of the Pro GBS potassium salt. In the PI-FAB experiment, Pro GBS is characterized by the potassium adduct of the molecular ion, i.e. $(MK+K)^+$, m/z 693

Glucobrassicin 7

To a solution of 1 mg ProGBS 6 in 1ml anhydrous methanol was added some drops of potassium methoxide (1N) until pH 8-9. The mixture was stirred 1h under inert atmosphere.The solvent was then removed by evaporation under a nitrogen flux to give GBS 7.

The absence of Pro GBS 6 was checked by HPLC. For this operation, an aliquot of the crude reaction mixture was evaporated to dryness and redissolved in HPLC mobile phase A. The

following step gradient elution was used : 100% A: , 5 min then 100% B: (flow rate : 1ml/min). Under these conditions, the retention times of glucobrassicin 7 and proglucobrassicin 6 were 6.5 and 17.8 min, respectively.

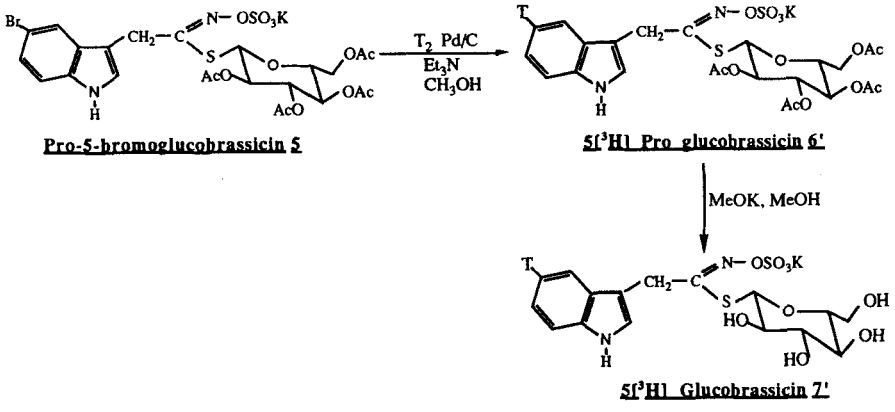
The analysis of the reaction mixture gave a unique chromatographic peak at 6.5 min, indicating that the deacetylation of 6 was complete after 1h.

GBS 7 was analyzed by FAB mass spectrometry (Figure 1).

As for Pro GBS, the FAB MS analysis of GBS 7 gave m/z 447,$(MK-K)^-$ (see fig.1), and m/z 525, $(MK+K)^+$, for the negative and positive ion experiments, respectively.

RADIOSYNTHESIS :

The reaction pathway for synthesizing 5 [^{3}H] glucobrassicin 7' is described in scheme 2.



Scheme 2 : Synthesis of 5 [^{3}H] glucobrassicin.

5[^{3}H] Proglucobrassicin 6'

1 mg of pro-5-bromoglucobrassicin 5, 1.4 mg Pd/C (18%), 5 µl Et_3N and 50 µl MeOH were stirred under 972 mbar of tritium gaz during 15 min. Catalyst was removed by filtration.The residual was dissolved in ethanol.The bulk radioactivity of the solution thus obtained was 57 mCi/ 50 ml ethanol.

The purification of compound 6' was carried out by HPLC (mobile phase B: flow rate 0.8 ml/ min) and 6' was located by counting each fraction before collecting on a fraction collector.

5 [^{3}H] Glucobrassicin 7'

The deacetylation of 5[^{3}H] proglucobrassicin 6' has been achieved according to the same procedure as for Pro GBS 6.

The radiopurity of 5[^{3}H] glucobrassicin 7' was checked by radio-HPLC (Step gradient

elution as for 7 preparation).

The compound 5[^{3}H] glucobrassicin 7' was analysed by negative FAB mass spectrometry (see Figure 1).

RESULTS AND DISCUSSION

Several sets of conditions were investigated for the catalytic reduction of 5:

Firstly, in the removal of halogen from an organic molecule by hydrogenolysis, the hydrogen halide formed poisons the catalyst, if not neutralized. For neutralization purposes we have used triethylamine which has been said to be superior to potassium hydroxide (11) previously used in the synthesis of [^{3}H] indol-3-yl carbinol (5).

Secondly, the sulfur-containing moiety is also known (12) to be a potential catalyst poison. However, by using a large catalyst excess (16 mg of 10% Pd/C) compound 5 can be converted to 6 over a period of 3h with 75% purity. Optimal H_2 pressure conditions were found to be 5 bars.

However, concerning the labelling experiments, it should be noted that (i) large catalyst load tends to yield poor specific activity products and (ii) the tritiation procedure must be conducted at atmospheric pressure, as pointed out by Rosen *et al.* (13).

Under the conditions described in the experimental section, tritiation of 5 in the presence of 25% Pd/C results in a conversion of 5 to 6' with 51% of radiopurity, determined by radio-HPLC analysis (mobile phase B, flow rate 1ml/min, retention time of 6' : 8.7 min).

6' was purified by HPLC and fraction collection.

The radiopurity of 5[^{3}H] Proglucobrassicin 6' thus purified was 98% and its HPLC retention time was directly superposable to the unlabelled compound 6 with UV detection at 235 nm.

The deacetylation of purified 6' leads to 98% of 5[^{3}H] GBS 7' (Scheme 2). The radio-HPLC analysis of 7' gave a single radioactive peak of retention time 6.5 min.

The specific activity was estimated to 14 Ci/mmole (based on limits of UV detection).

For comparative purposes, the mass spectrum of 5[^{3}H] GBS is presented in figure 1 together with that of unlabelled GBS .

As it was expected, the signal corresponding to $(MK-K)^-$ was shifted from m/z 447 to m/z 449, corresponding to the replacement of an hydrogen by a tritium atom. The high radiopurity of the synthetized 5[^{3}H] glucobrassicin is confirmed on figure 1b by the absence of signal at m/z 447.

Note that the spectrum shown in figure 1a was obtained from 2 µg of GBS disposed on the FAB target whereas figure 1b represents the spectrum obtained from 1 µg (estimated from the radioactivity) of 5[^{3}H] GBS. Despite this fact, it appears clearly that the mass spectrometric

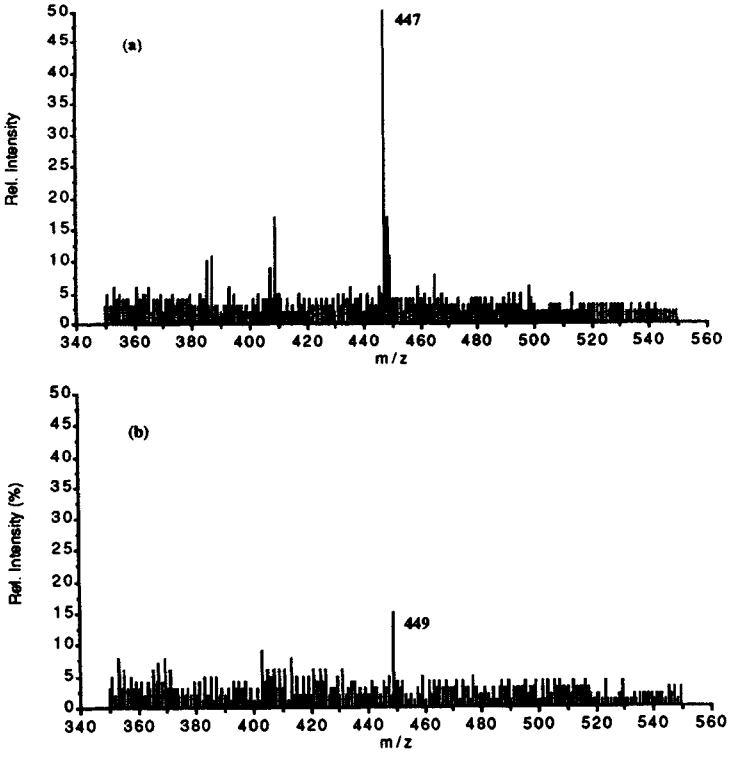


Figure 1 : FAB Mass spectra of (a) Glucobrassicin 7 and (b) 5[^{3}H] Glucobrassicin 7'

response was highly hindered in the case of the labelled compound. This was likely due to the contamination of the sample by a significant amount of HPLC buffer salts remaining after purification. Indeed, this was also expected since it is a well-know phenomenon in FAB mass spectrometry (14).

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