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Efficient reversed-phase purification of a hydrophobic reaction product following Mitsunobu-mediated glycosylation

Mani S. Iyer, Martin Palomo, Kevin M. Schilling, Yongping Xie, Leo Formanski, David E. Zembower*

MediChem Research, Inc., 2501 Davey Road, Woodridge, IL 60517, USA

Abstract

The Mitsunobu reaction was used to attach tetra-*O*-benzyl-D-glucopyranose to a monoindolylmaleimide, providing a key intermediate in the total synthesis of indolocarbazole topoisomerase I poisons. Using normal-phase silica gel chromatography, purification of the glycosylated product normally required multiple columns, resulting in poor recovered yields. Reversed-phase chromatography was used successfully to purify this highly hydrophobic material, rapidly and in high yield.

Keywords: Mitsunobu reaction; Glycosylation; Enzymes; Glucopyranoses; Maleimides; Indolocarbazoles; Alkaloids

1. Introduction

Topoisomerase I (Topo I) is an enzyme that catalyzes the topological state of DNA, and is essential during cellular events that can lead to disruption of DNA integrity, such as transcription, replication, and recombination. The key role of Topo I is to maintain DNA topology by catalyzing a single-stranded breakage of a phosphodiester bond, allowing free rotation of the intact strand around the break, providing relaxation of supercoiled DNA in steps of one linking number [1]. Subsequent enzyme-catalyzed religation of the nicked strand completes the catalytic cycle. Topo I is an important target for cancer therapy, and is the biological target of two clinically-used antitumor agents, camptosar and hycamtin, both analogues of the natural product camptothecin [2].

Recently, several research groups have demonstrated that indolocarbazole alkaloids represent a unique class of Topo I poison. Most notably, ED-110 [3] and NB-506 [4], both of which are semi-synthetic analogues of a microbial natural product, were each shown to exhibit potent antitumor activity in vitro and in vivo. We [5] and others [6] showed that both Topo I poisoning and antitumor activity can be attenuated through changes in ring substitution and differential positioning of hydroxyl groups. A clear structure–activity relationship has yet to be defined.

In the total synthesis of indolocarbazole Topo I poisons, the necessary *N*-glycosidic bond has been formed using both the Keonigs–Knorr reaction (see Ref. [7]) and, more recently, the Mitsunobu reaction (see Ref. [8], Fig. 1). The Mitsunobu reaction was originally adapted to the synthesis of indolocarbazole antitumor agents to allow regioselective introduction of the glycosidic moiety onto one specific indolic nitrogen. The optimized reaction conditions involved 1.0 equivalent of the bromoindolomaleimide **1**, and 3.0 equivalents each of tetra-*O*-benzyl-D-glucopyran-

*Corresponding author.

E-mail address: dzembower@medichem.com (D.E. Zembower).

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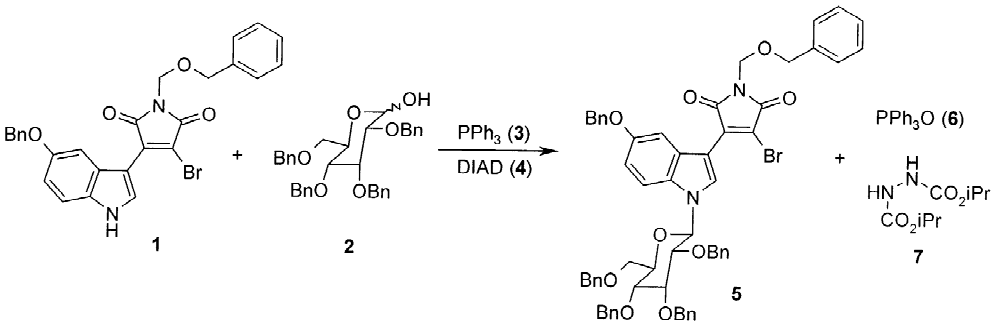

Fig. 1. Structures of substrates and products in the Mitsunobu-mediated glycosylation of bromoindolomaleimide **1**.

ose (mixture of α- and β-anomers, **2**), triphenylphosphine (PPh_3, **3**), and diisopropylazodicarboxylate (DIAD, **4**).

In our hands, the Mitsunobu-mediated glycosylation was extremely efficient on small scale (up to 5 g). However, because of the nature of the Mitsunobu reaction, and its generation of a significant quantity of by-products and unreacted starting materials [in this case, 1 equivalent of triphenylphosphine oxide (**6**), 1 equivalent of 1,2-bis(isopropoxycarbonyl)-hydrazine (**7**), and 2 equivalents each of unreacted triphenylphosphine, DIAD, and tetra-*O*-benzyl-D-glucopyranose], silica gel chromatography became unwieldy at larger scales. In this particular example, assuming the reaction proceeded to completion, the theoretical product mass only represents 29.6% of the total crude product mass.

2. Experimental

Reagents and solvents were purchased from commercial vendors and used without further purification. 1-*N*-Benzyloxymethyl-3-bromo-4-(5-benzyloxyindolo)maleimide (**1**) was prepared [9] via *N*-alkylation of 3,4-dibromomaleimide with benzyloxymethylchloride. Pre-packed reversed-phase cartridges were purchased from Biotage (Charlottesville, VA, USA), and purification was conducted with a Biotage 150M unit. HPLC analysis was conducted with a Zorbax SB-CN column (250×4.6 mm, 5 μm), eluting with MeCN–water (3:1) at a flow-rate of 1.0 ml/min, using UV detection at 215 nm.

*2.1. Preparation of 2-bromo-3-[5-benzyloxy-1-(2,3,4,5-tetra-O-benzyl-β-D-glucopyranosyl)-1H-indol-3-yl]-N-benzyloxymethylmaleimide (**5**)*

Into a cooled, oven-dried 3-l three-neck flask was placed **1** (23.64 g, 45.7 mmol), 2,3,4,5-tetra-*O*-benzyl-D-glucopyranose (**2**, 74.0 g, 137 mmol) and triphenylphosphine (**3**, 35.9 g, 137 mmol) and tetrahydrofuran (THF) (1.0 l) under N_2, and the solution was cooled to −78°C. Diisopropylazodicarboxylate (**4**, 27.7 g, 137 mmol) was added dropwise, maintaining the temperature at −78°C, and then stirred for 3 h. The solution was warmed to 0°C with the aid of an ice-water bath and stirring was continued for 2 h. The mixture was diluted with EtOAc (1200 ml), washed with HCl, brine, and water. The organic layer was dried over Na_2SO_4, filtered and concentrated to afford 174.9 g of crude product.

2.2. Chromatographic procedure

The purification was performed on a Biotage 150M apparatus, using a Biotage 150M reversed-phase column (KP-C18-HS, 35–70 μm, 60 Å, 500–550 m^2/g, 18% carbon load, endcapped, 30 cm×150 mm I.D.). The quantity of stationary phase in the column was 3.9 kg. The column was equilibrated by flushing sequentially with 4 l of 100% MeCN, 6 l of water–MeCN (50:50), and 6 l of water–MeCN (90:10).

The crude product mixture was divided in half, as two chromatographic runs were necessary to com-

plete the purification with a 150M column. Approximately 88 g of crude mixture was dissolved in 100 ml of dimethyl sulfoxide (DMSO), then injected onto the column. The column was eluted with the following gradient: 10 l of MeCN–water (10:90); 5 l of MeCN–water (30:70); 5 l of MeCN–water (50:50); 5 l of MeCN–water (60:40); 10 l of MeCN–water (70:30); 5 l of MeCN–water (80:20); 5 l of MeCN–water (90:10); 10 l of 100% MeCN; 5 l of MeCN–MeOH (98:2); column wash consisting of 20 l of MeOH–MeCN (20:80).

Fractions were collected in 250 ml portions. The desired product eluted at the 100% MeCN and MeCN–MeOH (98:2) stages. The column was re-equilibrated and the same gradient profile was performed on the second half of the material. The overall yield for this purification was 40.8 g (80.4%); m.p.; ^{1}H nuclear magnetic resonance (NMR) (C^2HCl_3) δ 3.64–4.02 (m, 7 H), 4.24 (dd, 1 H, J=10.2, 2.1 Hz), 4.58 (q, 2 H, J=12.0 Hz), 4.67 (m, 3 H), 4.92 (m, 3 H), 5.17 (m, 3 H), 5.34 (dd, 1 H, J=8.7, 2.7 Hz), 6.73 (m, 2 H), 6.98–7.08 (m, 4 H), 7.21–7.41 (m, 24 H), 7.48–7.55 (m, 4 H), 7.98 (d, 1 H, J=6.6 Hz); Fourier transform (FT) IR 3030, 2868, 1713, 1086, 1074 cm^{-1}. Anal. calculated for $C_{61}H_{55}BrN_2O_4 \cdot 2H_2O$: C, 68.09; H, 5.34; N, 2.60. Found: C, 68.20; H, 5.35; N, 2.22.

3. Results and discussion

The Mitsunobu-mediated glycosylation of **1** to form **5** proceeded to completion. However, the crude Mitsunobu reaction product required multiple silica gel column chromatographies to provide pure product. In general, triphenylphosphine (**3**), triphenylphosphine oxide (**5**), and DIAD (**4**) were readily separated from the desired product using silica gel chromatography, but the 1,2-bis(isopropoxycarbonyl)hydrazine (**6**) and, especially, the unreacted tetra-*O*-benzyl-D-glucopyranose (**2**), were very difficult to remove, due to similar retention factors on silica gel. Because of this tedious, extensive purification, isolated yields of the desired product **5** were inconsistent, but consistently low. Attempts to carry partially purified material on to the next synthetic step resulted in much lower yields at that stage.

To increase the isolated yield of **5**, it was necessary to identify a purification method that easily removed the excess tetra-benzylated sugar. Though a variety of purification methods were attempted (normal-phase chromatography using different solvent systems, trituration of the crude reaction mixture with various solvent mixtures, and recrystallization), none were successful.

During routine analysis of the crude reaction mixture on an analytical reversed-phase HPLC column, it was noted that the desired product exhibited a much longer retention time than all the other components in the mixture (Fig. 2). Though reversed-phase chromatography is often used in the purification of polar materials, it was not obvious that reversed-phase purification could be utilized for a highly hydrophobic material such as **5**.

As reported in Table 1, all the unreacted starting materials and reaction by-products eluted early on a reversed-phase analytical column, while the desired product eluted last. HPLC analysis of the crude reaction mixture at 215 nm (Fig. 2) showed presence of excess DIAD (**4**, t_R=3.36 min), tetra-*O*-benzylglucopyranose (**2**, t_R=4.16 min), 1,2-bis(isopropoxycarbonyl)hydrazine (**6**, t_R=5.12 min), and the desired product **5** (t_R=8.19 min). The starting material **1** was completely consumed, consistent with thin-layer chromatography (TLC) results. The triphenylphospine (**3**) and triphenylphosphine oxide (**7**) did not appear on the chromatogram due to low UV absorbance at 215 nm. In transitioning to a useful scale separation, we examined the utility of prepacked reversed-phase cartridges available from Biotage. A 150M cartridge contains 3.9 kg of C_{18} material in a convenient, sealed 30 cm×150 mm cartridge.

The Mitsunobu-mediated preparation of compound **5** was conducted twice, at scales of 43 and 45.7 mmol (23.0 and 23.6 g of starting **1**, respectively). Because of the quantity of by-products and unreacted starting materials present at this scale, the crude products were halved, and two chromatographic runs were required for each lot. In both cases, following a standard aqueous workup, one half of the concentrated crude reaction mixture was applied directly to the reversed-phase Biotage cartridge and eluted with an aqueous acetonitrile gradient, ramping from an initial 10% acetonitrile system to a 100% acetonitrile system, followed by a methanol–acetonitrile (10:90)

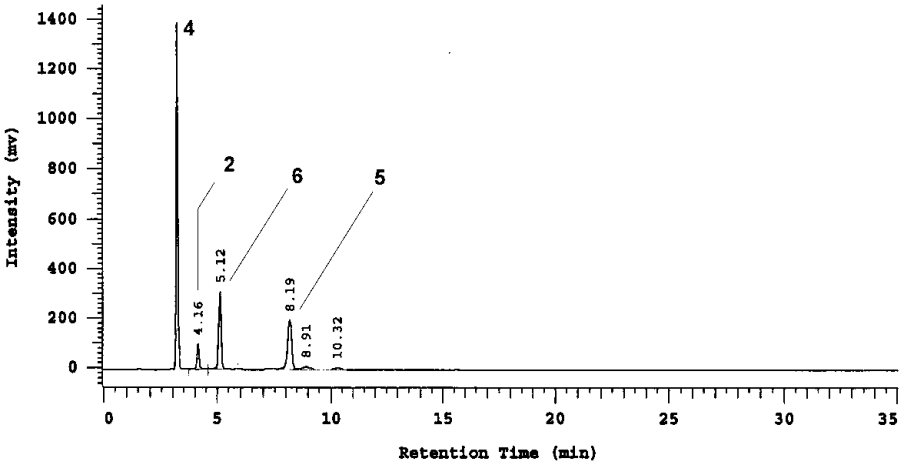

Fig. 2. HPLC chromatogram of crude Mitsunobu reaction mixture. HPLC analysis was conducted with a Zorbax SB-CN column (250×4.6 mm, 5 μm), eluting with MeCN–water (3:1) at a flow-rate of 1.0 ml/min, using UV detection at 215 nm.

column wash. The crude material was halved due to the quantity of material relative to the quantity of stationary phase in the Biotage cartridge. The desired product **5** eluted across the 100% acetonitrile and methanol–acetonitrile (10:90) fractions. The same column was used for all four chromatographic runs, without any observable decrease in chromatographic performance. For the two lots prepared in this study, the isolated yields of purified **5** were 75.0 and 80.4%, respectively.

4. Conclusions

The Mitsunobu reaction is an effective method for the mild introduction of protected sugar moieties, and was applied to the preparation of an intermediate for the synthesis of indolocarbazoles, which are under preclinical evaluation as antitumor agents. Because commercial tetra-*O*-benzyl-D-glucopyranose exists as a mixture of α- and β-anomers, excess sugar reagent (3.0 equivalents) was necessary in the preparation of **5**, which selectively provided the desired β-anomer of the product. This led to the presence of large quantities of unreacted reagents, including 2 equivalents each of unreacted sugar, triphenylphosphine, and DIAD, as well as the reaction by-products typical of a Mitsunobu reaction.

Reversed phase chromatographic separation is routine in the purification of polar compounds, but is not an obvious method for the purification of highly hydrophobic materials such as **5**. While standard normal-phase silica gel chromatography did allow purification of **5**, that process was extraordinarily labor intensive, and required multiple chromatographic runs that resulted in low to moderate isolated yields. Use of reversed-phase chromatography allowed purification of the crude Mitsunobu reaction product with only one chromatographic run, with reproducibly good yields.

Table 1
Retention times of reaction components on the Zorbax SB-CN reversed-phase analytical HPLC column

Component	Retention time (min)
1	4.05
2	4.16
3	4.45
4	3.36
5	8.13
6	5.12
7	4.29

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