Nanogram-scale derivatization of hydroxy groups for highly sensitive HPLC/MS/CD detection

Ning Zhao,[a] Jin-Song Guo,[a] Lee-Chiang Lo,[a] Nina Berova,[a] Koji Nakanishi,*[a] Garner T. Haupert,[b] M. Warrack[c] and Adrienne A. Tymiak*[c]

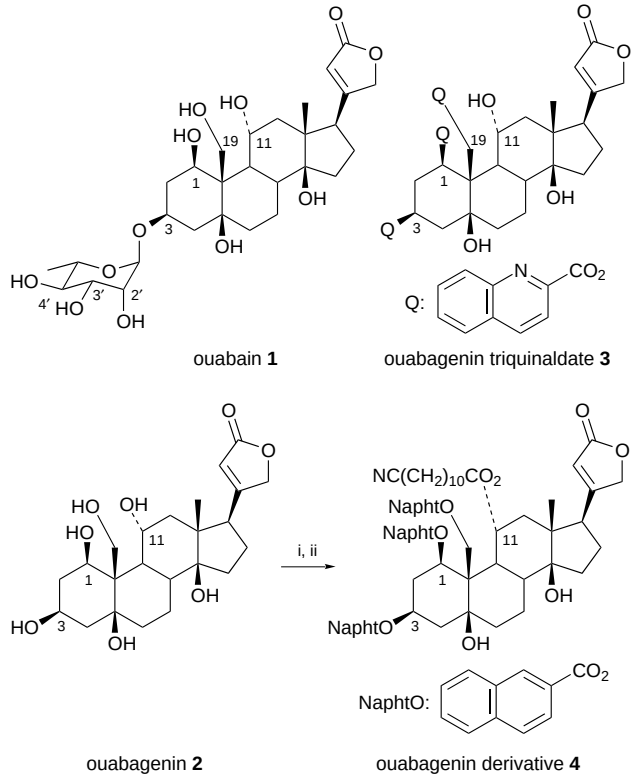
[a] *Department of Chemistry, Columbia University, New York, NY 10027, USA*
[b] *Renal Unit, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA*
[c] *Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 4000, Princeton, NJ 08543-4000, USA*

A strategy for performing submicrogram-scale structural studies of saponins and related compounds is worked out by: (*i*) naphthoylation to sensitize HPLC detection by fluorescence as well as configurational studies by exciton coupled CD; and (*ii*) ω-cyanoundecanoylation to increase LC/MS sensitivity (*ca*. 100-fold).

The specific binding of plant-derived digitalis glycosides by mammalian Na,K-ATPase (sodium pump) raised the possibility that a mammalian analogue of digitalis might exist. Hamlyn *et al*. reported a molecule from human plasma which by mass spectrometry (MS), chromatographic behaviour, *etc*., was undistinguishable from the plant saponin ouabain **1** (ouabain-like compound or OLC).[1] We had also isolated from bovine hypothalamus an inhibitor of the Na,K-ATPase (hypothalamic inhibitory factor or HIF). Similar to ouabain, HIF had an l-rhamnoside moiety but sub-μg scale characterizations showed that it was some isomer of ouabain.[2] Since rhamnosidic saponins are unknown as natural products from mammalian sources, it became important to compare HIF, OLC and ouabain **1** directly. This was performed by converting 300–400 ng of each into their penta-2-naphthoates upon which it was found that the HPLC retention times of HIF and OLC pentanaphthoates were identical but differed from that of ouabain 2′,3′,4′,1,19-pentanaphthoate. Most uniquely, although ouabain **1** exhibited a typical positively split exciton couplet in its circular dichroic spectrum (CD) at 235 nm, *i.e.* 245 nm (Δε +209 m^{-1} cm^{-1})/229 nm (Δε −170), those of HIF and OLC were devoid of any clear Cotton effects (!), thus showing that the arrangements of the five naphthoates are such that the spatial interactions are intramolecularly cancelled.[3] In summary, HIF and OLC are in all likelihood identical but differ from ouabain **1**.

Subsequent rhamnosidation/naphthoylation studies of ouabagenin and CD measurements of the pentanaphthoates coupled with CD computations† suggest the possibility that the genins of HIF and ouabain **1** may indeed be different. Because of the extremely limited amount of the mammalian cardiotonic factor(s) available from natural sources (*e.g.* 5 kg of bovine hypothalamus yields 1 μg of HIF),[3] our strategy is to hydrolyse HIF with naringinase, determine the genin structure by highly sensitive methods, and eventually synthesize the genin and attach the rhamnose. The final structure will be compared with the natural sample by HPLC and CD of its pentanaphthoate and bioassay of the saponin.

In the following, we describe two-step derivatizations of hydroxy groups leading to facile HPLC collection, and increased LC/MS and CD sensitivities. The reaction conditions are chosen to perform partial derivatization with the fluorescent naphthoate chromophores, and this is followed by further acylation of the less reactive hydroxy group with an MS sensitivity-enhanced moiety. Since all properties of HIF are very similar to those of ouabain **1**, suggesting the close similarity (or identity?) of the two genins, ouabagenin is employed as a model. In our previous studies, the HIF hydroxy groups were converted into naphthoates because of many favourable attributes:[4] high derivatization yield (90–95%), fluorescence, intense UV absorption and hence favourable chromophore for exciton coupled CD,[5,6] *etc*. Because a positively charged nitrogen atom greatly enhances the sensitivity in ionspray MS, we first sought to employ 2-quinaldates, with UV properties similar to those of 2-naphthoates, thus simplifying the interpretation of exciton coupled CD, *i.e.* λ_{max} of quinaldate in acetonitrile at 235 nm (ε 37 000 m^{-1} cm^{-1}) *cf*. naphthoate at 234 nm (ε 58 000). Ouabagenin 1,3,19-triquinaldate **3**, which was prepared in a high-yield microscale reaction using conditions similar to those described for the naphthoate (see below), gave an intense split CD slightly weaker than that of the trinaphthoate: quinaldate **3**,‡ UV λ_{max} in acetonitrile at 235 nm (ε 109 000 m cm^{-1}); CD 231 (Δε +125), 241 nm (−186), *A* (amplitude) −311, *cf*. trinaphthoate, λ_{max} in acetonitrile at 231 nm (ε 147 000); CD 227 (+250), 240 nm (−292), *A* −542. Although the MS sensitivity of triquinaldate **3**



Scheme 1 One-pot/two-step reaction (naphthoylation and 11-cyanoundecanoylation) of ouabagenin **2**. *Reagents*: i, 2-naphthoylimidazole, DBU, MeCN; ii, $[NC(CH_2)_{10}CO]_2O$, DBU, DMAP.

was enhanced, the quinaldate approach was abandoned because of the much weaker fluorescence.

Compound **4** was designed to overcome the disadvantages of **3**. In a one-pot/two-step reaction (Scheme 1), naphthoylation of ouabagenin was followed by 11-cyanoundecanoylation to give the strongly fluorescent **4**, 85% yield; 231 nm (ε 158 000 m cm^{-1}); CD 227 (+260), 240 (−317), A −577; λ_{ex} = 234 nm, λ_{em} = 360 nm).§ A typical reaction is performed as follows. Ouabagenin (*ca.* 1 μg, dried in a silylated vial), was dissolved in 120 μl anhydrous acetonitrile, and treated with 2-naphthoylimidazole (3 mg) and DBU (0.4 μl). The reaction mixture was stirred for 1 h at room temperature followed by the addition of 11-cyanoundecanoic anhydride (5 mg),¶ and a catalytic amount of DMAP. After stirring for a further 8 h at room temperature, the reaction was quenched by adding 1 ml of 1 : 4 acetonitrile–water. After removal of acetonitrile, the mixture was applied to a Waters C_{18} SepPak cartridge, and then washed sequentially with acetonitrile–water mixtures: 10 ml of 1 : 4, 10 ml of 2 : 3, 8 ml of 4 : 1 and 1 ml of 9 : 1. The final product was eluted with 4 ml acetonitrile, loaded onto a Vydac C_{18} column (4.6 × 250 mm, 10 μm) and eluted isocratically with acetonitrile–water 82 : 18 at 1 ml min^{-1}. The products were detected by a fluorescence detector (Hewlett Packard 1046A fluorescence detector, λ_{ex} = 234 nm, λ_{em} = 360 nm). As depicted in Fig. 1(*a*), the HPLC peaks of ouabagenin trinaphthoate **3** overlap with the asterisked naphthoylation reaction byproducts, whereas the tetranaphthoate peak does not; introduction of the hydrophobic undecanoyl chain increases the retention time leading to no overlap [Fig. 1(*c*)]. Furthermore, **4** offered increased LC/MS detectability.‖ Whereas the limit of detection (LOD) for ouabagenin trinaphthoate was *ca.* 25 ng by single ion monitoring ionspray LC/MS, as little as 300 pg of acyl derivative **4** could be detected under the same conditions. Only part of the sensitivity enhancement observed with **4** is accounted for by increased hydrophobicity since LODs of 0.7 and 0.3 ng were measured for ouabagenin tetranaphthoate and 11-undecanoyl ouabagenin trinaphthoate, respectively.

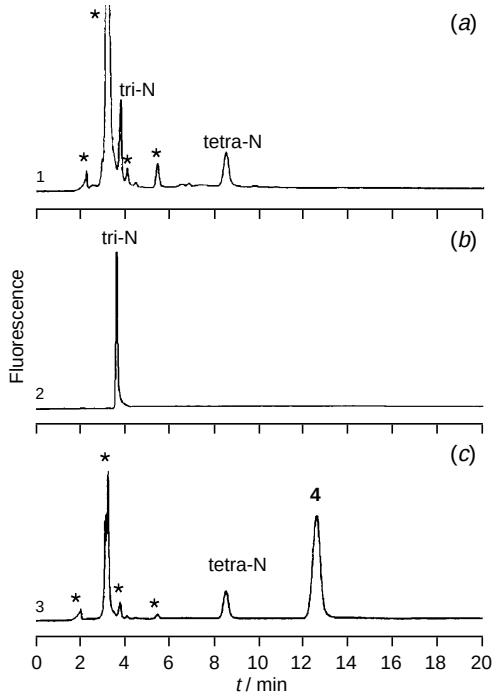


Fig. 1 Fluorescence-detected HPLC profiles: (*a*) reaction mixture of naphthoylation of ouabagenin; (*b*) authentic ouabagenin trinaphthoate; (*c*) reaction mixture of one-pot/two-step derivatization. Asterisked peaks (*) are from reagents. tri-N = ouabagenin trinaphthoate; tetra-N = ouabagenin tetranaphthoate.

In summary, a general sub-μg derivatization/HPLC/MS/CD approach has been developed. The intense and fluorescent UV chromophore increases the sensitivity of fluorescence-detected HPLC and CD measurements, while the cyanoundecanoyl side chain increases the hydrophobicity to facilitate HPLC/MS analysis as well as increasing the MS sensitivity by *ca*. 100-fold as compared to a substrate without nitrogen. Application of this one-pot/two-step approach in HIF characterization is under way. It is clear that, with some modification, this approach is applicable to the identification, characterization, *etc.*, of other hydroxylated compounds.

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Footnotes

† J. Dong, I. Akritopoulou-Zanze, J. Guo, N. Berova, K. Nakanishi and G. Haupert, in preparation.

‡ ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (2 H, t, *J* 8.4 Hz), 6.97 (1 H, d, *J* 8.4 Hz), 5.89 (1 H, s, H-22), 5.87 (1 H, d, *J* 11.7 Hz, H-19), 5.71 (2 H, br s, H-1, H-3), 4.95 (1 H, d, *J* 18.2 Hz, H-21), 4.78 (1 H, d, *J* 11.7 Hz, H-19), 4.31 (1 H, m, H-11); FABMS 905 (M^+).

§ **4** exhibited the following characteristic peaks in ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (1 H, s), 8.31 (1 H, s), 8.07 (1 H, s), 6.49 (1 H, s, H-1), 5.89 (1 H, s, H-22), 5.70 (1 H, br s, H-3), 5.33 (2 H, br s, 2 × H-19), 5.19 (1 H, m, H-11), 4.90 (1 H, d, *J* 17.8 Hz, H-21), 4.77 (1 H, d, *J* 17.8 Hz, H-21); FABMS 1093 (M^+).

¶ 11-Cyanoundecanoic anhydride was prepared from 11-cyanoundecanoic acid and DCC.

‖ Ionspray LC/MS was conducted on a Waters Nova-Pak C18 column (3.9 × 150 mm) with 1 ml min^{-1} isocratic flow of 20% 2 mm NH_4OAc in MeCN interfaced to a SCIEX API III mass spectrometer, using a 20 : 1 flow split and single-ion monitoring at the *m/z* of the $[M + NH_4]^+$ ion.

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