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Structure of 'endogenous ouabain'*

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Abstract: The structural characterization of the ouabain-like sodium pump inhibitor in mammals (so-called 'endogenous ouabain') has long been hindered by the paucity of the sample material. While many microscale structural analyses, including LC/MS and sugar analysis, could not differentiate 'endogenous ouabain' from ouabain, our past nanogram-scale 'pentanaphthoylation' followed by HPLC and circular dichroic spectroscopy (CD) had thought to have distinguished the two. 'Endogenous ouabain' has since been considered as a subtle structural isomer of ouabain. However, further search for endogenous ouabain has now shown that this is not the case. The factors responsible for the unsuccessful characterization for several decades were a series of unexpected reactions, including formation of mixtures of ouabain borates and glycerol naphthoates. The in vivo species giving rise to the reported biological activities of hypothalamic inhibitory factor (HIF) remains to be clarified in full.

Cardenolides constitute a unique group of steroids, which exhibit specific inhibitory activity against Na^+,K^+-ATPase (the sodium pump) in mammalian and insect cells. The chemical structures of cardenolides are characterized by 3β-OH (or 3β-O-sugar), C/D–*cis*-ring juncture, 14β-OH, and 17β-unsaturated lactone ring [1]. Although they have been mostly purified from plants and toads, the conserved and specific binding sites for cardenolides in mammalian sodium pump led to the speculation that there might exist the mammalian counterparts of cardenolides, which control the physiological functions of sodium pump [2,3].

The search for the endogenous Na^+,K^+-ATPase inhibitor in mammals over the past 30 years have led to conflicting reports stating that the factor is ouabain (a plant-origin cardiac glycoside) or not ouabain [2–6]. The chemical and physiological characterizations of this so-called 'endogenous ouabain' has been hindered by the extremely limited sample quantity available from mammalian tissues. This has led to the numerous contradictory reports on the structure and physiology of this enigmatic compound [7–22].

The chemical structure of the endogenous ouabain from bovine hypothalamus, also called hypothalamic Na^+,K^+-ATPase inhibitory factor (HIF), has been examined by a series of microscale structural analyses [23]. HIF and the aglycone of HIF, which had been obtained through naringinase (α-L-rhamnosidase) treatment, exhibited identical LC/MS profiles with ouabain and ouabagenin, respectively. The identity of the sugar moiety of HIF was further confirmed as L-rhamnose by acid sugar cleavage followed by chiral GC/MS analysis. While these structural data indicated that HIF was ouabain itself, many reported biological activities of HIF, which were distinctly different from those of ouabain [17–22], still left the possibility that the aglycone of HIF and ouabain may be slightly different. Subsequently, HIF and ouabain were submitted to a nanogram-scale naphthoylation protocol, in which the products were analyzed by reversed-phase(RP) HPLC and CD spectroscopy (Fig. 1) [23,24]. The naphthoylation products of HIF turned out to be different from ouabain 1,19,2′,3′,4′-pentanaphthoate (ouabain PN), the major naphthoylation product of ouabain, the former HIF product eluting slightly earlier than ouabain PN on RP-HPLC. In addition, it gave a 'zero-CD' profile, which was totally different from the positive CD couplet of ouabain PN. Subsequently, endogenous ouabain from human plasma, named ouabain-like

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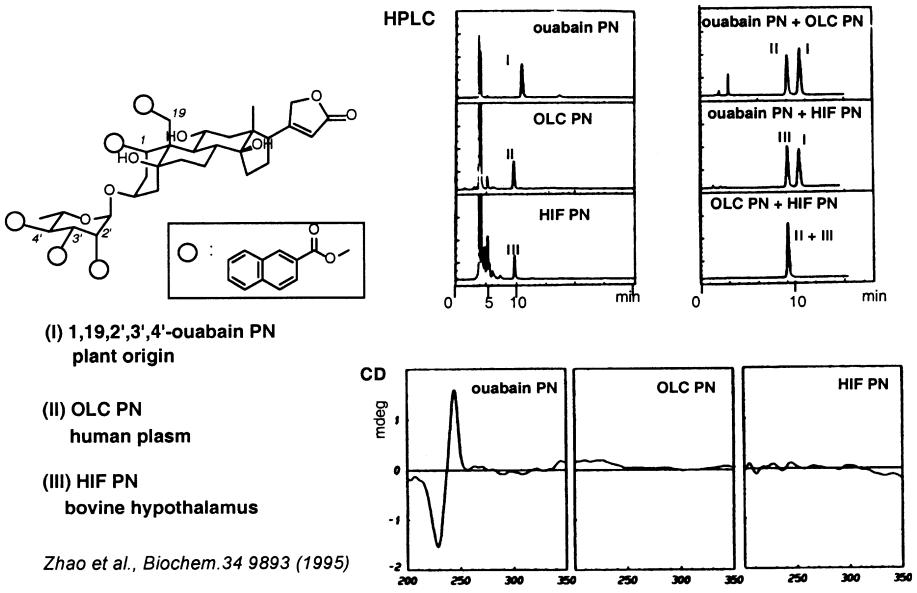

Fig. 1 Naphthoylation appeared to enhance structural characteristics of ouabain, OLC and HIF.

compound (OLC), was also submitted to the naphthoylation protocol and gave the same product as that from HIF [24]. These naphthoylation results led to a widely accepted notion that endogenous ouabain is a close structural isomer of plant ouabain [2,3,5,25]. It was, however, a daunting task to identify endogenous ouabain out of the large number of possible ouabain isomers. A theoretical CD study was then attempted to screen sugar position isomers of ouabain, in which CD of all possible pentanaphthoates of the sugar position isomers were calculated to see whether any one of them showed the 'zero-CD' profile (Figs 1 and 2) [26]. The validity of the CD calculation was corroborated by the agreement between the calculated values and experimental values of synthetic analogs. While this study provided valuable information regarding theoretical CD calculations of conformationally flexible molecules, none of the pentanaphthoates derived from rhamnose position isomers reproduced the 'zero-CD' profile of endogenous ouabain derivative (Fig. 2).

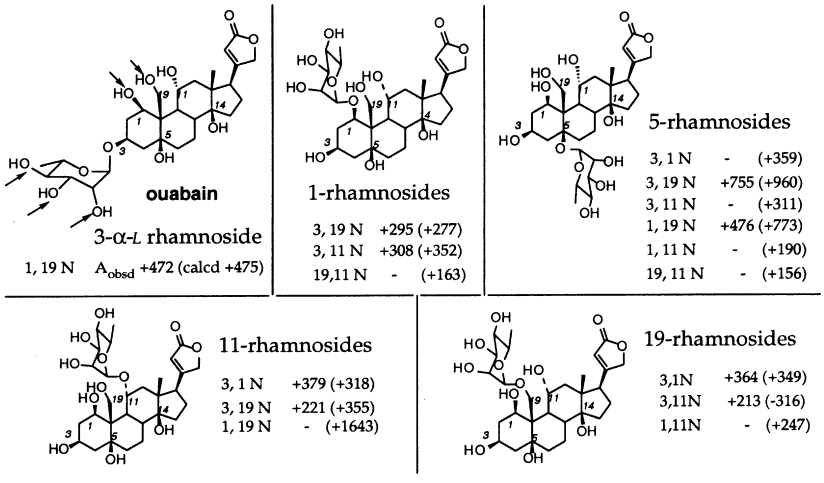

Fig. 2 Amplitudes of exciton couplets of ouabagenin pentanaphthoates, observed and calculated (π-electron SCF-CI dipole velocity MO method).

Meanwhile, an intensive effort to purify HIF for further structural studies led to the purification of 14 μg (1996) and 3 μg (1997) of new samples from bovine hypothalamus. These larger sample quantities of endogenous ouabain made it possible to perform ^{1}H-NMR measurements, which gave two totally different spectra for HIF (Fig. 3). The earlier of the two samples, 14 μg-HIF (1996), showed two sets of ^{1}H-NMR signals with a ratio of approximately 3:2, which can be seen in the high field (≈ 0.8 p.p.m.) and the low field (≈ 5.9 p.p.m.) regions. None of the ^{1}H-NMR signals of this 14 μg-HIF matched those of ouabain, which further supported the conclusion drawn from the earlier naphthoylation studies on HIF and OLC [23,24]. On the other hand, the subsequently purified sample, 3 μg-HIF (1997), exhibited a different ^{1}H-NMR profile, containing ouabain-like signals as well as some other unknown peaks.

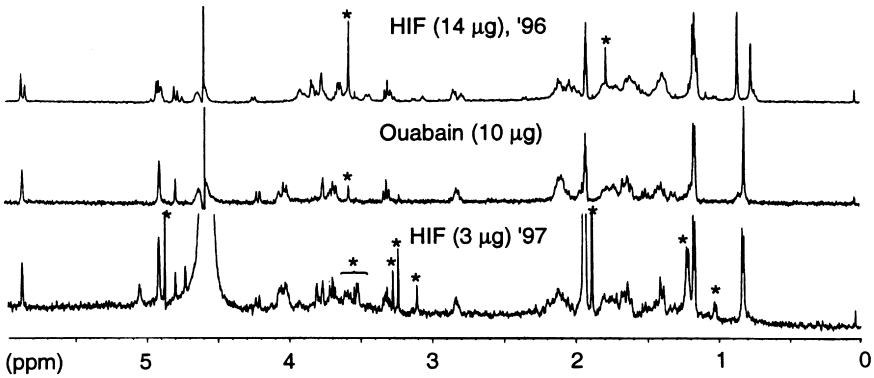

Fig. 3 ^{1}H-NMR of 1996 HIF, 1997 HIF samples and ouabain.

These ^{1}H-NMR measurements, however, eventually led to a totally unexpected finding, which clarified the ambiguities associated with the past structural analyses on HIF and OLC [27,28]. The ^{1}H-NMR profile of 14 μg-HIF (1996) was found to be reproduced upon storage of ouabain in glass vial for overnight, which in turn could be simulated by a mixture of ouabain and sodium tetraborate (Fig. 4). On the other hand, the signals in the 3 μg-HIF (1997) spectrum turned out to arise from a mixture of ouabain and ouabain-trigonal borate (Fig. 5), which can be prepared from ouabain and boric acid. The two purified HIF samples were therefore in fact ouabain, which was converted into the two borates after purification in borosilicate glasswares. The purification protocols used for the preparation of the two HIF samples were then reviewed, and it was found that both samples were collected and dried in borosilicate glass tubes. Since 0.05% TFA was used in the final RP-HPLC purification step for the preparation of the 3 μg-HIF (1997) [27,28], it had given a mixture of ouabain and ouabain trigonal borate. On the other hand, the 14 μg-HIF (1996) was purified without TFA, which led to the formation of ouabain tetrahedral borate

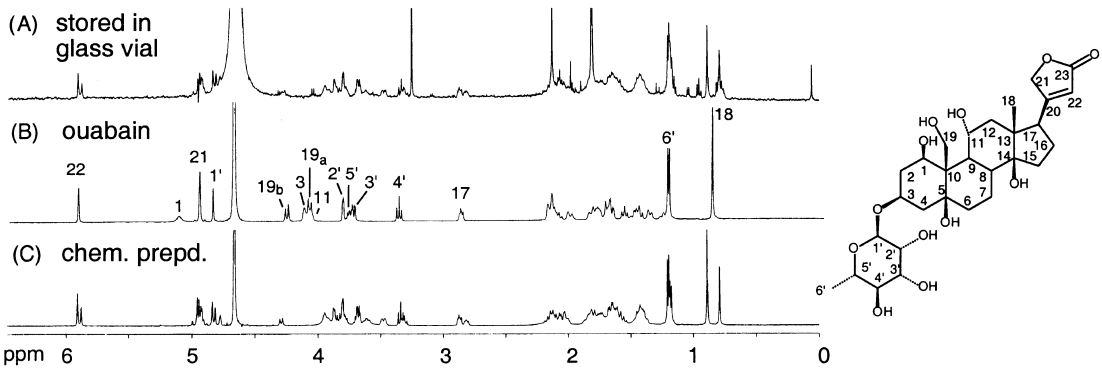

Fig. 4 ^{1}H-NMR of (A) aqueous solution of ouabain (10 μg) that had been stored in a borosilicate vial; (B) ouabain; and (C) ouabain borate prepared from ouabain and sodium tetraborate.

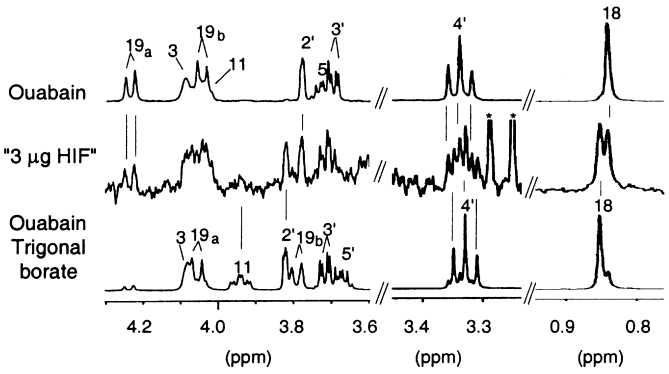

Fig. 5 NMR of ouabain, HIF (1997) and chemically prepared ouabain and boric acid (1:1) in water followed by lyophilization.

complexes. The full structures of ouabain tetrahedral borate complexes were established by a series of NMR and MS studies. It turned out that the polyhydroxylated ouabain backbone moiety serves as polydentate ligand to tetrahedral borate in neutral to basic pH, and gives rise to two interconverting coordination isomers of ouabain tetrahedral borate complexes (Fig. 6). The borate attachment site in the trigonal borate complex has not been identified; however, the hydroxyls of the aglycone moiety are most likely involved in the borate coordination since NMR of the 3 μg HIF (Fig. 2) could be reproduced upon acid treatment of the ouabain tetrahedral borate sample (Fig. 6), in which 1,5,19- and 1,11,19-OH groups are involved in the borate complexation.

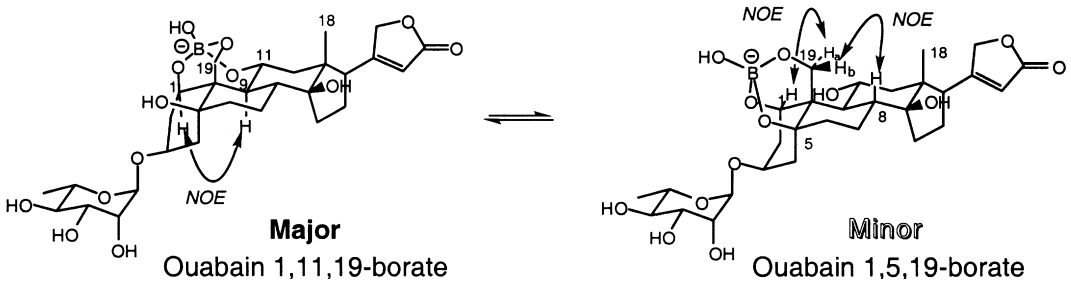

Fig. 6 Structures of two interconverting ouabain tetrahedral borates.

Although masking of the hydroxyl groups in the borates explained why ouabain PN was not formed in the past nanogram-scale naphthoylation studies on HIF and OLC [23,24], the identity of the 'zero-CD' product remained unclear. It was subsequently, in retrospect accidentally, found that this 'zero-CD' product was not a derivative of 'endogenous ouabain' but glycerol trinaphthoate (!) [27,28], which seemed to arise from nanogram-level contamination of glycerol in the original HIF and OLC samples. Past naphthoylation studies [23,24], were misled by the absence of ouabain PN as well as by the conspicuous byproduct from glycerol contamination.

The propensity of ouabain molecule to form various borates points to a possibility that 'endogenous ouabain' exists as some unknown complexes in the physiological environment. As exemplified by the ouabain borate complexes (Fig. 6), the flexibility of A/B rings and hydroxyl groups of the ouabain molecule allows ouabain to bind to a variety of inorganic species and macromolecules existing in the mammalian body. The numerous contradictory accounts on the detection, purification, and biology of endogenous ouabain [7–22] may also have been, at least in part, caused by such unknown complexes of ouabain.

It is to be noted that the 1,5,11,19-hydroxyl groups, which are involved in the ouabain-borate complexation, are not required for inhibition of the sodium pump. The finding of the large structural

distortion caused by the borate complexation leads to the possibility that the spatial locations of 14-OH, the C-3 rhamnose, and C-17 butenolide, all important for sodium pump inhibition, can be modulated through these hydroxyl groups. In other words, it may be possible to alter the biological profiles of ouabain by changing its conformation through the flexible hands (1,5,11,19-OH groups) on the molecule [28]. A variety of fixed conformers of ouabain analogs are currently being prepared, and their physiological properties are being studied with a cell-based assay system using a microphysiometer (Cytosensor^{TM}), which can detect the extracellular acidification rate, an index for cellular metabolic activity. Ongoing studies is aimed at clarifying the interaction between ouabain and the sodium pump on a molecular structural basis with the help of a series of conformationally fixed ouabain analogs, each having slightly different locations of the biologically important functional groups. Further attempts to fully clarify the nature of the *in vivo* species is also underway.

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