Na,K-ATPase Inhibitors from Bovine Hypothalamus and Human Plasma Are Different from Ouabain: Nanogram Scale CD Structural Analysis[†]

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ABSTRACT: The specific, high affinity binding of plant-derived digitalis glycosides by the mammalian sodium and potassium transporting adenosine triphosphatase (Na,K-ATPase, or sodium pump), a plasma membrane enzyme with critical physiological importance in mammalian tissues, has raised the possibility that a mammalian analog of digitalis might exist. We previously isolated and structurally characterized from bovine hypothalamus a novel isomer of the plant glycoside, ouabain, which differs structurally only in the attachment site and/or the stereochemistry of the steroid moiety [Tymiak et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 8189—8193]. Hamlyn and co-workers reported a molecule purified from human plasma which by mass spectrometry could not be distinguished from plant ouabain [Hamlyn et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 6259—6263]. Since rhamnoside cardiotonic steroids are not known as natural products from mammalian sources, it became important to compare these two pure isolates to determine if the same or structurally distinct compounds had been found. Our results indicate that the human and bovine Na,K-ATPase-inhibitors are identical, but different from plant ouabain. This supports the notion that the human sodium pump may be under specific physiological regulation by a mammalian analog of the digitalis glycosides.

Experimental evidence linking an endogenous digitalis-like Na,K-ATPase inhibitor to fluid and electrolyte homeostasis through regulation of renal sodium excretion and, in the case of dysregulated states, to the pathogenesis of a prevalent human disease, hypertension, stimulated efforts to isolate and characterize such a compound (Haber and Haupert, 1987; Goto et al., 1992). Using affinity chromatography combined with HPLC we purified a bovine hypothalamic Na,K-ATPase inhibitor factor, HIF[1] (Tymiak, 1993). It has a molecular weight of 584, making it isobaric with ouabain. HIF was then found to be an α-L rhamnoside, as is ouabain, while reversed-phase HPLC failed to separate HIF from ouabain. However, physiological and biochemical assays demonstrated that the biological effects of HIF were similar but not identical to the pharmacological inhibitor, ouabain (Haupert, 1994). Presumably, these apparent differences in biological properties were due to subtle structural differences not detectable by either mass spectroscopy or HPLC coinjection. In order to cope with the submicrogram amounts of available HIF, we developed a combined

microderivatization/HPLC/circular dichroic (CD) procedure to enhance the structural difference between HIF and ouabain. In this approach, the multiple hydroxyl groups are naphthoylated in 90—95% yield to give strongly fluorescent naphthoates with intense absorption maximum (Ikemoto, 1992); for ouabain penta-1,19,2′,3′,4′-naphthoate the ϵ at 234 nm is 232 000. The strong absorption is a particularly important attribute, since other factors being equal, the amplitude of the characteristic exciton split Cotton effects are proportional to the square of ϵ (Harada and Nakanishi, 1983; Nakanishi and Berova, 1994). The sensitivity for HPLC detection of pentanaphthoates is at the ca. 1 pmol level by UV detection and 1 order of magnitude higher by fluorescence detection. Application of this method led to the first clear-cut evidence that HIF differs from ouabain using a total of 300 ng (0.51 nmol) of sample.

Independently, Hamlyn and co-workers reported isolation of a Na,K-ATPase inhibitor from diafiltrates of human plasma (Hamlyn et al., 1991). By using fast atom bombardment (FAB) mass spectroscopy, they concluded that the molecular mass of the plasma inhibitor was identical to that of ouabain. Fragmentation patterns after exhaustive acetylation were also indistinguishable from plant ouabain. In addition, limited physiological testing of the purified isolate also gave results indistinguishable from ouabain (Bova et al., 1991), leading the authors to conclude that the ouabain-like compound (OLC) from human plasma might indeed be ouabain.

However, identification of the sugar and aglycon moieties of OLC could not be achieved by the spectroscopic methods employed. A crucial aspect to be clarified before charac-

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[1] Abbreviations: HIF, hypothalamic Na,K-ATPase inhibitory factor; CD, circular dichroic; OLC, ouabain-like compound; Na,K-ATPase, sodium and potassium transporting adenosine triphosphatase [EC 3.6.1.37].

terization of the full structures of HIF and/or OLC was a nonambiguous comparison of HIF, OLC, and ouabain. The availability of ca. 2 μg of pure OLC allowed direct comparison of the two mammalian-derived Na,K-ATPase inhibitors with ouabain.

MATERIALS AND METHODS

Materials. The extraction and purification of HIF and OLC from bovine hypothalamus and human plasma, respectively, have been previously described in detail (Tymiak et al., 1993; Hamlyn et al., 1991). Briefly, HIF was obtained from methanol/water extracts of bovine brain tissue quick-frozen on dry ice at the time of tissue collection and maintained at −80 °C until processing. Delipidation was achieved by petroleum ether and chloroform extraction, with subsequent lipophilic gel chromatography in methanol, ion-exchange chromatography, and further concentration using CHP20P resin (MCI gel, Mitsubishi Chemical, Tokyo, Japan). HIF was further purified by an affinity step employing SDS-extracted canine kidney Na,K-ATPase coupled to paramagnetic iron particles (Bio Mag 4100, Advanced Magnetics, Cambridge, MA) via glutaraldehyde cross linking of primary amino groups. Affinity-purified HIF was concentrated as a single compound with reversed-phase C_{18} HPLC using a linear gradient of acetonitrile/water. The maximum yield was 1 μg of purified HIF from 5 kg of bovine hypothalamus.

OLC was adsorbed from diafiltrates of human plasma obtained at the time of plasmapheresis using an Amberlite XAD-2 column. The methanol eluate was fractionated by preparative-scale reversed-phase HPLC. The partially purified OLC was subjected to affinity extraction using partially purified lamb kidney Na,K-ATPase in a batch technic. Supernatants containing OLC dissociated from the enzyme−inhibitor complexes were purified to a single compound with reversed-phase HPLC. Treatment of 300 L of human plasma yielded 31 μg of pure OLC (Ludens et al., 1991). Ouabain was purchased from Sigma.

Naphthoylation and HPLC Analysis (Figures 1 and 2). HIF, OLC, and ouabain were naphthoylated under conditions described previously except for a minor but important change (see below) and purified by HPLC. Each sample of HIF ($\sim$300 ng), OLC ($\sim$400 ng), and ouabain ($\sim$400 ng) was dried in a silylated vial separately. Naphthoylation was performed by dissolution in 190 μL of anhydrous acetonitrile, followed by addition of naphthoylimidazole (1.5 mg) and 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.4 mL) (Ikemoto et al., 1992). The reaction mixture was stirred for 2 h at room temperature and then quenched by adding 1 mL of an acetonitrile/water (1:4) solution; previously, the mixture was stirred for 3 h but this was shortened to 2 h, which ensured that the sole product was a single pentanaphthoate, that is, no trace of hexanaphthoate. After acetonitrile was removed under reduced pressure, the mixture was applied to a Waters C_{18} SepPak cartridge. The cartridge was washed sequentially with the following acetonitrile/water mixtures: 10 mL of 1:4, 10 mL of 2:3, and 5 mL of 1:1. The naphthoylated products were eluted with 5 mL of acetonitrile and then subjected to a Vydac C_{18} column (4.6 × 250 mm, 10 mm) with isocratic elution of acetonitrile/water 82:18 at 1 mL/min. The products were detected by a fluorescence detector (Shimadzu RF-551 detector, λ_{ex} = 234 nm, λ_{em} = 374 nm).
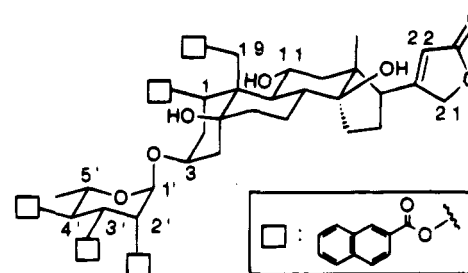
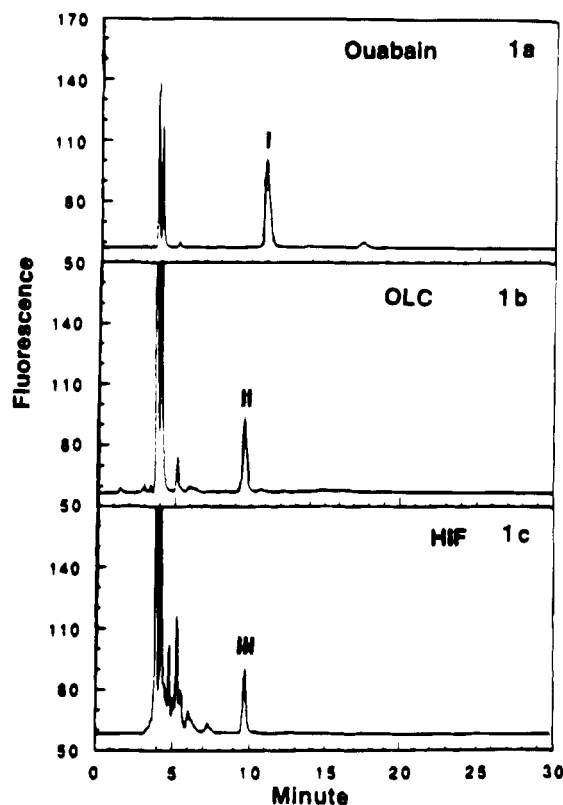**1, 19, 2', 3', 4'-ouabain-pentanaphthoate**

FIGURE 1: Fluorescence-detected HPLC profiles for crude naphthoylation reactions. Column and elution were as described in the Materials and Methods: (a) Ouabain reaction (peak I, ouabain pentanaphthoate); (b) OLC reaction (peak II, OLC pentanaphthoate); (c) HIF reaction (peak III, HIF pentanaphthoate).

The same HPLC conditions were used for the coelution of HIF, OLC, and ouabain pentanaphthoates.

Under the HPLC conditions described above, the polynaphthoates of ouabain could be clearly separated because of the large differences in retention times: tetranaphthoates all eluted at ca. <6 min as base line separated peaks (unpublished results), the pentanaphthoate at 10 min, and the 1,-11,19,2',3',4'-hexanaphthoate at 24 min (unpublished results). The ouabain 1,19,2',3',4'-pentanaphthoate (Figure 1a, peak I) exhibited the following characteristic proton peaks in the ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.32 (1H, s, H-1), 5.85 (1H, s, H-22), 5.78 (1H, bs, H-2'), 5.66 (1H, dd, J = 10.12, 10.12 Hz, H-4'), 5.44 (1H, dd, J = 3.06, 10.12 Hz, H-3'), 5.35 (1H, s, H-1'), 5.31 (2H, ABq, J = 12.08, 22.44 Hz, 2 × H-19), 4.92 (1H, d, J = 18.04 Hz, H-21), 4.76 (1H, d, J = 18.04 Hz, H-21), 4.50 (1H, bs, H-3), 4.11 (2H, m, H-5', H-11). As reported previously (Tymiak et al., 1993), the minuscule amount of HIF pentanaphthoate (Figure 1c, peak III) led to great difficulties in FAB-MS identification of the
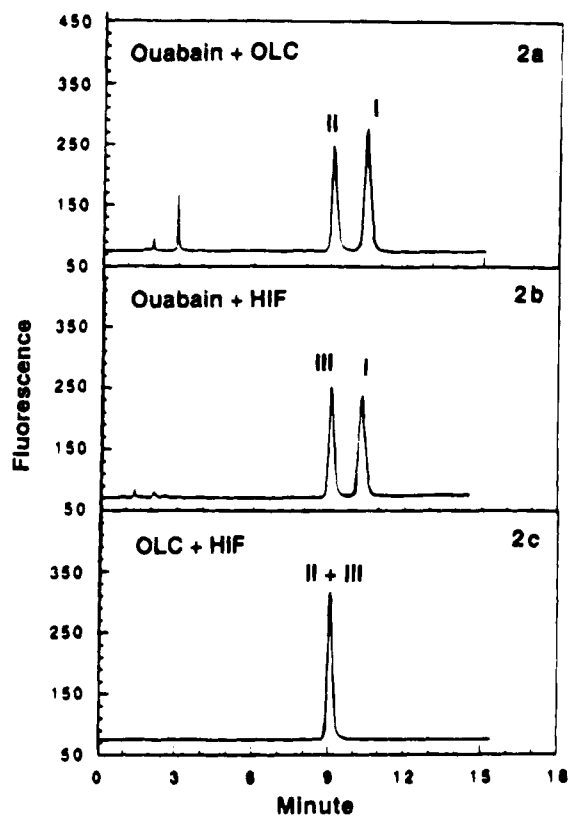
FIGURE 2: Fluorescence-detected HPLC profiles for coinjected ouabain pentanaphthoate I, with OLC pentanaphthoate II (a) and HIF pentanaphthoate III (b); (c) coinjection of pentanaphthoate of OLC and HIF. Column and elution were as described in the Materials and Methods.

M^+ peak at 1354 which was detected as a very weak peak with signal/noise = 2 using the highly sensitive JEOL HX110-110 instrument (Suntory Institute for Bioorganic Research, Osaka, Japan). The present OLC naphthoate (Figure 1b, peak II) was not submitted to FAB-MS measurements in order to save precious material; however, judging from the identical retention time with HIF pentanaphthoate (Figure 2c, peak III) it is clear that we are dealing with the pentanaphthoate of OLC.

CD Spectroscopy (Figure 3). CD spectra (in acetonitrile) of HPLC-purified ouabain pentanaphthoate, OLC pentanaphthoate, and HIF pntanaphthoate were obtained using a JASCO J-720 spectropolarimeter. Ouabain pentanaphthoate λ_{max} 233 nm, ϵ 232 000 in acetonitrile, shows a typical exciton split CD centered at 235 nm, i.e., 245 nm ($\Delta\epsilon$ +209)/ 229 nm ($\Delta\epsilon$ −170).

RESULTS

Figure 1 shows the HPLC profiles of naphthoylation products of the three inhibitors. As expected, under the experimental conditions employed ouabain yields only the ouabain 1,19,2′,3′,4′-pentanaphthoate (Figure 1a, peak I) which was confirmed by coinjection with authentic ouabain 1,19,2′,3′,4′-pentanaphthoate. Derivatives of OLC and HIF show similar HPLC retention times with major product peaks II and III (Figure 1b,c), respectively, eluting earlier than ouabain pentanaphthoate (Figure 1a, peak I). Coinjection of peaks I and II and I and III showed in a clear-cut manner that OLC and HIF were both different from ouabain (Figure 2a,b). This is the first evidence to show that OLC differs from ouabain. In contrast, under these HPLC conditions, coinjection of OLC and HIF displays only a single peak (Figure 2c).

Figure 3 shows the CD spectra (in acetonitrile) of HPLC-purified ouabain pentanaphthoate (Figure 3a), OLC pentanaphthoate (Figure 3b), and HIF pentanaphthoate (Figure 3c). Ouabain 1,19,2′,3′,4′-pentanaphthoate gives a split CD curve with positive Cotton effect reflecting exciton coupling among the various naphthoate groups (Harada and Nakanishi, 1983), whereas exciton split Cotton effects among chromophores within the OLC and HIF derivatives are internally cancelled leading to an absence of signal and reflecting spatial arrangement different from ouabain.

DISCUSSION

Although underivatized OLC, HIF, and ouabain cannot be separated by HPLC, significantly, their pentanaphthoates prepared by nanogram scale derivatization and purification show different retention times.
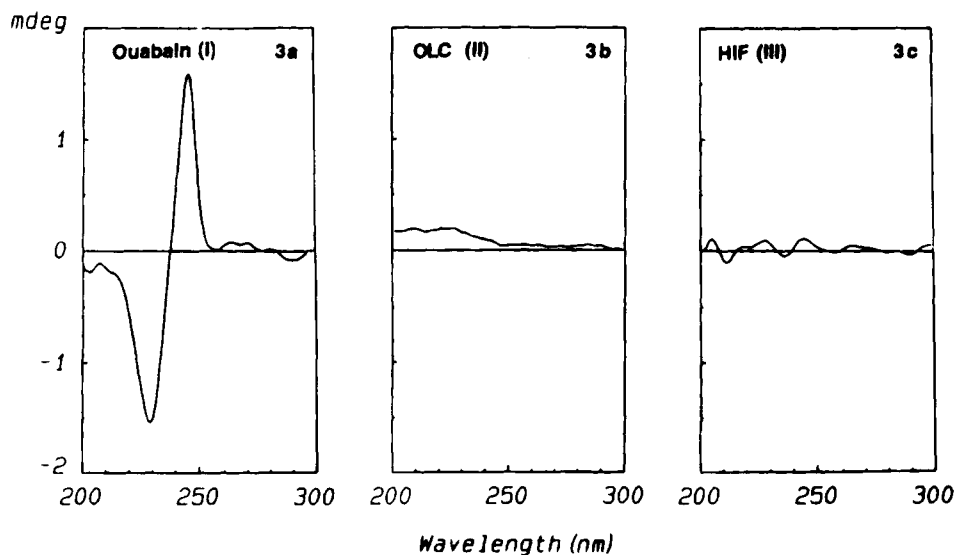

FIGURE 3: CD spectra (in acetonitrile) of HPLC-purified ouabain pentanaphthoate (a), OLC pentanaphthoate (b), and HIF pentanaphthoate (c). mdeg, millidegrees.

Naphthoylation products of OLC and HIF (II, III) also exhibit CD spectra strikingly different from that of ouabain pentanaphthoate (I) (Figure 3). Despite the low concentration of ca. 4×10^{-7} M, ouabain pentanaphthoate still shows its characteristic positively split exciton-couplet (Figure 3a). In contrast, at similar concentration as estimated from the UV absorbance, the CD of OLC and HIF naphthoates show no distinct Cotton effects (Figure 3b,c). While the data do not prove that HIF and OLC are identical, the absence of any Cotton effects in both spectra indicates that the five naphthoate chromophores are interacting in a manner that leads to intramolecular cancellation of all couplings between the naphthoate chromophores. This is a very unique case, and we are not aware of examples in which molecules containing multiple chromophores, especially five as in the present case, exhibit CD curves with no exciton-split CD. However, the amplitudes of split CD curves vary dramatically with configurational changes even at a single stereogenic center as exemplified by the following amplitudes (or A values) of hexapyranose per-2-naphthoates: D-Glc $A = +238$, D-gal $+772$, D-man -675 (Golik et al., 1983). Therefore, it is quite possible that the interactions of the five naphthoates cancel out leading to CD with no conspicuous Cotton effects. However, it is difficult to imagine that there could be two different chiral arrangements of chromophores in OLC and HIF which both lead to complete Cotton effect cancellations. In conjunction with identical retention times, the lack of circular dichroism strongly suggest that HIF and OLC are identical, but different from ouabain.

In order to exclude the possibility that the mammalian OLC and HIF are products of ouabain isomerization occurring during the isolation process, ouabain was submitted to the isolation/purification/naphthoylation protocol employed for OLC and HIF. HPLC and CD analysis showed no structural changes had occurred, thus eliminating any possibility of OLC and HIF being artifacts of ouabain.

Much effort has been expended to identify and characterize inhibitors of Na,K-ATPase that could have physiological and/or pathophysiological roles (Haber and Haupert, 1987; Tamura et al., 1988, 1994; Goto et al., 1990). Previously, in spite of intense studies, highly purified OLC and ouabain behaved identically in biochemical (Hamlyn et al., 1989), chromatographic (Ludens et al., 1991), immunologic (Harris et al., 1991), and mass spectral analyses (Mathews et al., 1991). The present study represents at least two major advances in this regard. Firstly, OLC and ouabain are not identical. Secondly, the present study provides evidence that HIF and OLC are identical. Whether HIF and OLF are truly the product of de novo mammalian biosynthesis is not yet known. Studies of rat hypothalami suggest that HIF, or a closely related molecule, may be endogenous. Extracts of Milan strain rat hypothalami, purified by the same method as used for bovine hypothalamus, yield Na,K-ATPase inhibitory activity which coelutes with HIF on HPLC (Ferrandi et al., 1992). The finding in these studies that Milan genetically hypertensive rats with elevated plasma levels of Na,K-ATPase inhibitory activity have 10-fold more inhibitor in the hypothalamus than Milan normotenive control rats, despite identical housing and diet for both groups, supports an endogenous origin for the hypothalamus-derived Na,K-ATPase inhibitor in this species. Whether bovine HIF and human plasma OLC are structurally identical to the rat hypothalamic inhibitor is not known. Arguments for an endogenous origin for OLC have been put forward (Hamlyn et al., 1991).

Structural identity is striking because OLC and HIF were isolated from different species and different tissues, human plasma and bovine hypothalamus, thus suggesting the possibility that this unique molecule may exist widely in mammals and that the human sodium pump may be specifically regulated by an endogenous mammalian analog of the plant-derived cardiac glycosides. Although HIF and OLC meet many of the criteria of a hormone, the exact role of such a system is not completely understood at present. Theories have been put forth in connection with salt balance and blood pressure control (DeWardener and MacGregor, 1980; Blaustein, 1993). Structure determination of this mammalian Na,K-ATPase inhibitor is in progress.

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REFERENCES

Blaustein, M. P. (1993) *Am. J. Physiol. 264*, C1367−C1387.

Bova, S., Blaustein, M. P., Ludens, J. H., Harris, D. W., DuCharme, D. W., & Hamlyn, J. M. (1991) *Hypertension 17*, 944−950.

DeWardener, H. E., & MacGregor, G. A. (1980) *Kidney Int. 18*, 1−9.

Ferrandi, M., Minotti, E., Salardi, S., Florio, M., Bianchi, G., & Ferrari, P. (1992) *Am. J. Physiol. 263*, F739−F748.

Golik, J., Liu, H.-W., Dinovi, M., Furukawa, J., & Nakanishi, K. (1983) *Carbohydr. Res. 118*, 135−146.

Goto, A., Ishiguro, T., Yamada, K., Ishii, M., Yoshioka, M., Eguchi, C., Shimora, M., & Sugimoto, T. (1990) *Biochem. Biophys. Res. Commun. 173*, 1093−1101.

Goto, A., Yamada, K., Yagi, N., Yoshioka, M., & Sugimoto, T. (1992) *Pharmacol. Rev. 44*, 377−399.

Haber, E., & Haupert, G. T., Jr. (1987) *Hypertension 9*, 315−324.

Hamlyn, J. M., Harris, D. W., & Ludens, J. H. (1989) *J. Biol. Chem. 264*, 7394−7404.

Hamlyn, J. M., Blaustein, M. P., Bova, S., DuCharme, D. W., Harris, D. W., Mandel, F., Mathews, W. R., & Ludens, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6259−6263.

Harada, N., & Nakanishi, K. (1983) *Circular Dichroic Spectroscopy— Exciton Coupling in Organic Stereochemistry*, University Science Books, Mill Valley, CA.

Harris, D. W., Clark, M. A., Fisher, J. F., Hamlyn, J. M., Kolbasa, K. P., Ludens, J. H., & DuCharme, D. W. (1991) *Hypertension 17*, 936−943.

Haupert, G. T., Jr. (1994) in *The Sodium Pump* (Bamberg, E., & Schoner, W., Ed.) pp 732−742, Steinkopff Darmstadt, New York.

Ikemoto, N., Lo, L.-C., & Nakanishi, K. (1992) *Angew. Chem., Int. Ed. Engl. 31*, 890−891.

Ludens, J. H., Clark, M. A., DuCharme, D. W., Harris, D. W., Lutzke, B. S., Mandel, F., Mathews, W. R., Sutter, D. M., & Hamlyn, J. M. (1991) *Hypertension 17*, 923−929.

Mathews, W. R., DuCharme, D. W., Hamlyn, J. M., Harris, D. W., Mandel, F., Clark, M. A., & Ludens, J. H. (1991) *Hypertension 17*, 930−935.

Nakanishi, K., & Berova, N. (1994) in *Circular Dichroism, Principles and Applications* (Nakanishi, K., Berova, N., & Woody, R. W., Ed.) pp 361−398, VCH Publishers, New York.

Tamura, M., Lam, T.-T., & Inagami, T. (1988) *Biochemistry 27*, 4244−4253.

Tamura, M., Harris, T. M., Phillips, D., Blair, I. A., Wang, Y. F., Hellerqvist, C. G., Lam, S. K., & Inagami, T. (1994) *J. Biol. Chem. 269*, 11972−11979.

Tymiak, A. A., Norman, J. A., Bolgar, M., DiDonato, G., Lee, H., Parker, W. L., Lo, L.-C., Berova, N., Nakanishi, K., Haber, E., & Haupert, G. T., Jr. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8189−8193.