PERGAMON

Phytochemistry 53 (2000) 7–12

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Secologanin synthase which catalyzes the oxidative cleavage of loganin into secologanin is a cytochrome P450

Hirobumi Yamamoto[a,b], Nobuyuki Katano[b], Ayaka Ooi[b], Kenichiro Inoue[b,*]

[a]*Laboratory in Medical Plants Garden, School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[b]*Department of Pharmacognosy, Gifu Pharmaceutical University, 6-1 Mitahora-higashi 5 chome, Gifu 502-8585, Japan*

Received 18 May 1999; received in revised form 18 August 1999

Abstract

Secologanin synthase, an enzyme catalyzing the oxidative cleavage of the cyclopentane ring in loganin to form secologanin, was detected in microsomal preparations from cell suspension cultures of *Lonicera japonica*. The reaction required NADPH and molecular oxygen, and was blocked by carbon monoxide as well as by several other cytochrome P450 inhibitors, indicating that the reaction was mediated by cytochrome P450. Of the substrates examined, only specificity for loganin was demonstrated. A possible reaction mechanism is described. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Lonicera japonica*; Caprifoliaceae; Cell suspension cultures; Iridoid glucoside; Secologanin; Secologanin synthase; Cytochrome P450; Enzyme; Biosynthesis

1. Introduction

Indole alkaloids such as reserpine, ajmaline, ajmalicine, vincristine and vinblastine are biosynthesized from tryptamine and secologanin, a secoiridoid glucoside (Cordell, 1974). These alkloids are distributed in the Apocynaceae, Rubiaceae, Loganiaceae and Nyssaceae, and many are of importance in medicine manufacture (Mann, Davidson, Hobbs, Banthorpe & Harborne, 1994). While Dagnino, Schripsema and Verpoorte (1995) have revealed that the ability to produce terpenoid precursors limits the biosynthesis of terpenoid indole alkaloids, the biosynthetic pathway to secologanin and the method for its production in high yield by plant cell suspension cultures has not yet been established. Especially, the mechanism for the formation of secologanin, a final terpenoid precursor in the biosynthesis of these alkaloids, from loganin remained unsolved, even though it involves a unique oxidative cleavage of the methylcyclopentane ring in the latter molecule. Previous biosynthetic studies (Inoue, Takeda, Tanahashi & Inouye, 1981; Battersby, Westcott, Glusenkamp & Tiezte, 1981; Inoue, Tanahashi, Inouye, Kuwajima & Takaishi, 1989) ruled out the possibility of ionic fission of the cyclopentane ring of two plausible precursors, 10-hydroxy- (Battersby, 1967) and 6-hydroxy-loganin (Inoue et al., 1989) leading to secologanin. Thus, at the present time, a direct cleavage of the cyclopentane ring of loganin via a radical or a hydride process seemed to be most likely as the mechanism for secologanin formation (Inoue et al., 1981; Battersby et al., 1981). However, although the overall ability to convert loganin into secologanin was demonstrated in cell suspension cultures of *Lonicera* species (Tanahashi, Nagakura, Inouye & Zenk, 1984), the precise enzymlogy involved concerning this conversion was not given, presumably owing to the low enzyme activity responsible for this fission.

In a previous paper (Yamamoto, Katano, Ooi & Inoue, 1999), it was demonstrated that cell suspension cultures of *L. japonica* lacked the capability of secologanin biosynthesis but had the ability to convert loganin into secologanin. In the present paper, we now report the detection and characterization of secologa-

* Corresponding author.

PII: S0031-9422(99)00471-9

Table 1
NADPH and loganin concentration dependencies of secologanin synthase in the microsomal fraction from *L. japonica* cell suspension cultures[a]

Substrate/cofactor	Concentration (mM)	Activity (pkat/mg protein)
NADPH	0.01	0.00
	0.10	0.07
	1.00	1.59
	10.00	1.48
Loganin	0.01	0.00
	0.10	0.30
	1.00	1.27
	10.00	1.12

[a] In each experiment, concentration of loganin or NADPH was fixed at 1 mM, respectively.

nin synthase, the enzyme catalyzing this conversion in *L. japonica* cultured cells.

2. Results and discussion

2.1. Detection and properties of secologanin synthase activity

There are some issues to be solved in establishing the quantitative formation of secologanin, following its generation from loganin through the action of secologanin synthase since it is unstable; it has a methyl ester group easily hydrolyzed by dilute alkali, an *O*-glucosyl group hydrolyzable by acid, and an active aldehyde group which easily forms a Schiff base with amines and can also react with protic solvents, such as methanol. Accordingly, the use of acid, alkali or protic solvents for termination of the enzyme reaction has to be avoided as far as possible. Furthermore, the quantitative extraction of secologanin from the reaction mixture with organic solvent is also difficult due to its high solubility in water. Indeed, even when *n*-BuOH was used, only ca. 30% of secologanin was recovered from the buffer solution. Therefore, in the present study, the enzyme reaction was terminated by chilling the reaction mixture on an ice–water bath and the resulting mixture was applied to a short Diaion HP-20 column. The eluate with EtOH was concentrated in vacuo and analyzed by reversed phase HPLC. Thus, when a crude cell-free extract or microsomal preparation of *L. japonica* cultured cells was incubated with 1 mM loganin and 1 mM NADPH at pH 7.5 for 2 h, a new peak with an UV absorption pattern identical to that of secologanin was detected by HPLC-photodiode array analysis. By direct comparison of the retention time and the FABMS spectrum of this product with that of authentic secologanin by LC-FABMS analysis, the enzyme product was identified as secologanin. The secologanin synthase activity in the microsomal fraction was more than 10 times higher than that in the crude cell-free extract, indicating that this enzyme activity was membrane-associated (data not shown).

The reaction catalyzed by secologanin synthase showed an optimum at pH 7.5 (data not shown). The optimum concentrations of loganin and NADPH for secologanin synthase were both 1 mM (Table 1). However, the curves of the concentration-reaction velocity did not fit with the Michaelis–Menten equation presumably due to high concentrations of loganin, NADPH and the enzyme preparation i.e. this being responsible either for the non-stationary state of the reaction or being due to Schiff's base formation between the aldehyde group of secologanin and the amino groups of proteins in the reaction mixture. In any event, at present, the enzyme activities were not detectable at lower concentrations of either loganin or NADPH. For this reason, the calculation of K_m and V_{max} values will require further experiments with the radioisotopically labeled substrate.

2.2. Participation of a cytochrome P450 on secologanin formation

The characterization of secologanin synthase was investigated in some detail using the microsomal protein preparation: NADPH was essential for enzymatic activity (Table 2). When NADPH was substituted with NADH, only 11.5% of the activity was detected, and none with FAD. The enzyme also required molecular oxygen for oxidative cleavage of the carbon–carbon bond of loganin (Table 3). When the reaction mixture was bubbled with N_2 gas to displace O_2, the measurable enzymatic activity decreased to 11% of that when air was used. An absolute requirement for oxygen was next demonstrated by removal of O_2 from the assay by addition of the glucose/glucose oxidase/catalase system

Table 2
Cofactor requirements of secologanin synthase in *L. japonica* cell suspension cultures

Cofactor	Activity (pkat/mg protein)	Relative activity (% of control)
NADPH (1 mM)	1.65	100.0
NADH (1 mM)	0.19	11.5
FAD (1 mM)	0.00	0.0

Table 3
Influence of different treatments on secologanin synthase activity from *L. japonica* cell suspension cultures

Treatment	Relative activity[a] (% of control)
Control (air)	100.0
N_2	11.5
Air + glucose + glucose oxidase + catalase	0.0
Air + glucose + boiled glucose oxidase + catalase	80.8
CO : O_2 (9 : 1) dark	51.1
CO : O_2 (9 : 1) light[b]	97.1

[a] Control activities (pkat/mg protein) for anarobiosis and CO inhibition were 3.41 and 5.12, respectively.
[b] White light (20,000 lx).

(Kochs & Grisebach, 1987) where all activity was inhibited completely. This same inhibition did not notably occur when glucose oxidase in the oxygen-consuming system was denatured by heating (81% of the control). Taken together, these results suggested that oxidative cleavage reaction of the C-7–C-8 bond of loganin was catalyzed by a cytochrome P450 enzyme. To confirm the participation of a cytochrome P450 catalyzed conversion on this cleavage reaction, the effects of carbon monoxide (Estabrook, Cooper & Rosenthal, 1963) and several known cytochrome P450 inhibitors (Lamberts, Bons, Bruining & de Jong, 1987; Wendorff & Matern, 1986; Rossi, 1983; Fujita, Oba & Uritani, 1982) were investigated. As shown in Table 3, the reaction was inhibited by CO/O_2 (9:1) in the dark and this inhibition could be reversed by illumination with white light. All cytochrome P450 inhibitors (0.1 mM) used in the present study, metyrapone, cytochrome c, miconazole and ancymidol, inhibited or partially inhibited the reaction (Table 4) unless the conditions employed, with ancymidol completely inhibiting formation of secologanin. However, KCN (Saunders, Holmes-Siedle & Stark, 1964) hardly affected the activity (Table 4). Based on these observations, it can be concluded that secologanin synthase belongs to the group of cytochrome P450 monooxygenases.

Table 4
Effects of cytochrome P450 inhibitors on secologanin synthase activity[a]

Inhibitor (0.1 mM)	Relative activity (% of control)
Control	100.0
Metyrapone	43.7
Cytochrome c	11.0
Miconazole	36.1
Ancymidol	0.0
KCN	79.4

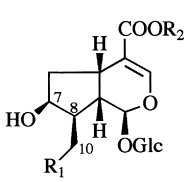
[a] In the control experiment, secologanin synthase activity was 2.54 pkat/mg protein.

2.3. Substrate specificity

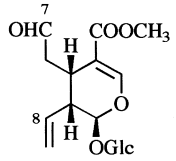
The substrate specificity for this cytochrome P450 enzyme was next examined using loganin, loganic acid, 10-hydroxyloganin, 7-epiloganin, 7-dehydrologanin, 7-dehydrologanic acid, 8-epiloganin and verbenalin, respectively, as potential substrates. Of the eight glucosides tested, only loganin was converted into any other product as detected by HPLC (data not shown); this product being secologanin as described earlier. Therefore, this P450 enzyme accepts only loganin as substrate, in agreement with results obtained earlier through the previous biosynthetic experiments (Inoue et al., 1981; Battersby et al., 1981).

In the present study, we have therefore provided the first definitive experimental evidence that the conversion of loganin into secologanin is mediated by a cytochrome P450-dependent monooxygenase presumably through a radical mechanism. Plant cytochrome P450 enzymes, which typically insert one oxygen atom into lipophilic compounds, are known to catalyze the oxidative reactions of various types (Sono, Roach, Coulter & Dawson, 1996; Halkier, 1996; Bolwell, Bozak & Zimmerlin, 1994). Among them, the hydroxylation associated with carbon–carbon cleavage involving aryl migration has been identified in the isoflavonoid, coumarin and steroid biosynthesis (Hakamatsuka, Hashim, Ebizuka & Sankawa, 1991). The formation of psoralen, a furanocoumarin, from (+)-marmesin, a hydroxyisopropyldihydrofuranocoumarin, is reasonably interpreted as the same reaction (Hakamatsuka et al., 1991). The conversion of loganin into secologanin can presumably be explained by a similar mechanism to that in the formation of psoralen as shown in Fig. 1. The abstraction of a hydrogen radical at C-10 of loganin occurs via cleavage of the carbon–carbon bond between C-7 and C-8 to form a carbon radical at C-7. This radical is then converted into an aldehyde group through hydroxylation (“a” route in Fig. 1) or desaturation (“b” route) to generate secologanin. Hakamatsuka and Sankawa (1997) proposed an alternative mechanism where the reaction starts with the successive abstraction of a hydrogen

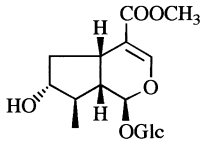
radical and an electron at C-10 of loganin to yield a carbonium ion which undergoes carbon–carbon cleavage through an ionic mechanism to afford secologanin as shown in "c" route of Fig. 1. Thus, the precise experimentally determined mechanism of this P450 reaction (associated with C–C bond cleavage in loganin) must await further inquiry.



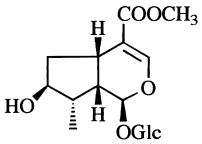
$R_1 = H, R_2 = CH_3$: loganin
$R_1 = H, R_2 = H$: loganic acid
$R_1 = OH, R_2 = CH_3$: 10-hydroxyloganin



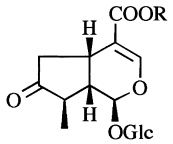
secologanin



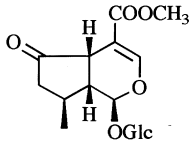
7-epiloganin



8-epiloganin



$R = CH_3$: 7-dehydrologanin
$R = H$: 7-dehydrologanic acid



verbenalin

Formulae

3. Experimental

3.1. Plant material and culture method

The origin and subculturing of cell suspension cultures of *L. japonica* were described previously (Yamamoto et al., 1999). Fresh cells (1.5 g) were transferred to 20 ml of Murashige–Skoog's liquid medium (Murashige & Skoog, 1962) containing 10 μM naphthaleneacetic acid and 10 μM 6-benzylaminopurine, and were agitated on a rotary shaker at a speed of 100 rpm at 23°C in the dark and subcultured every 2–3 weeks.

3.2. Enzyme preparation

For enzyme preparation, all procedures were carried out at 4°C. Ten-day-old cultured *L. japonica* cells (20 g) were homogenized in 40 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 10 mM DTT, 10% glycerol and 2 g PVPP by a teflon homogenizer. The homogenate was centrifuged at 12,000 g for 20 min. For the preparation of the microsomal fraction, the 12,000 g supernatant was centrifuged at 100,000 g for 30 min. The pellet was washed twice with the same buffer and then resuspended in 4 ml of the same buffer.

Protein content. Protein contents were determined according to the method of Ref. (Bradford, 1976).

3.3. Enzyme reaction

The incubation mixture contained 0.5 μmol loganin (100 mM Tris–HCl buffer, pH 7.5), 0.5 μmol NADPH (100 mM Tris–HCl buffer, pH 7.5) and enzyme extract, 400 μl in a total volume of 500 μl. The reaction was initiated by addition of loganin to the mixture and after the incubation for 120 min at 30°C, terminated by chilling the reaction mixture on an ice–water bath. The mixture was immediately passed through a 2 ml Diaion HP-20 short column equilibrated with H_2O to absorb the glucosides which were eluted with 3 ml of EtOH. The eluate was evaporated in vacuo to give the crude glucosides which were dissolved in 0.2 ml of EtOH containing 5 μg scopoletin as an internal standard and 40 μl of the solution was analyzed by HPLC.

For the investigation of the effects of different gaseous environments, test tubes (10 ml), on ice, containing all reaction components except NADPH were sealed with septum caps, and 200 ml of N_2, CO/O_2 (9:1) mixture or air were slowly bubbled through, respectively. The reaction was initiated by addition of 0.5 μmol NADPH in 50 μl of the same buffer. The reaction was stopped, treated and analyzed as described above. To examine the effect of the light on the inhibition by CO, the tubes were kept in the System Biotron (20,000 lx of fluorescent lights) for 120 min at 30°C. Tubes covered with aluminum foil were also kept in the same chamber as for the dark condition.

For the oxygen consuming experiments, 40 mM glucose, 50 unit glucose oxidase (Nacalai Tesque, Japan) and 105 unit catalase (Sigma, USA) were added to the enzyme solution containing 50 μmol loganin. After preincubation for 30 min at 30°C, the reactions were initiated by addition of 0.5 μmol NADPH.

3.4. HPLC analysis

The amount of secologanin was determined by reversed phase HPLC using Bio-Sil C18 HL 90-5 S column (5 μm, 4.6 × 250 mm, Bio-Rad) in an oven at 40°C, with CH_3CN/H_2O linear gradient solvent system, from 15 to 35% MeCN in 40 min at a flow rate of 0.5 ml/min, monitoring the absorption at 240 nm.

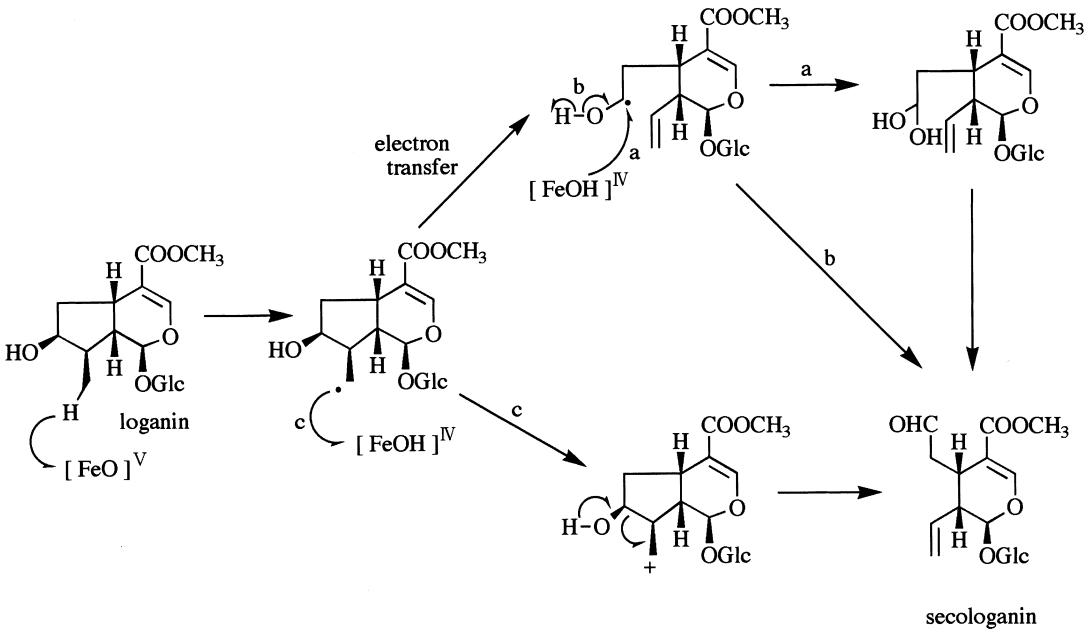


Fig. 1. Possible reaction mechanisms for the cytochrome P450-catalyzed oxidative cleavage of loganin to secologanin. The route "c" was proposed by Hakamatsuka and Sankawa (1997).

Quantities were calculated from the peak area at 240 nm recorded by Chromatopac C-RA4 (Shimadzu).

3.5. Identification of the reaction product

The identification of the product was carried out on the basis of UV and MS spectra obtained using a Shimadzu SPDM-6A HPLC-photodiode array system (Shimadzu, Japan) as well as a Shimadzu High Performance Liquid Chromatograph–Mass Spectrometer LCMS-QP8000. The HPLC condition used in the LC–MS system was as follows: column, Shim-pack VP-ODS (5 μm, 2.0 × 150 mm); solvent, CH_3CN/H_2O; linear gradient solvent system, from 10% MeCN to 30% MeCN in 30 min at a flow rate of 0.2 ml/min, monitoring the absorption at 240 nm; oven temperature, 40°C; Rt. of secologanin, 8.5 min. Negative FAB-MS *m/z*; 387 (M-1).

Acknowledgements

We are grateful to Professor Y. Takeda, Faculty of Integrated Arts and Sciences, The University of Tokushima, for a generous gift of 8-epiloganin and to Ms. K. Arakawa, Kyoto Analytical Applications Center, Shimadzu Corporation for the measurement of LC-MS spectrum. Financial support by Japanese Bioindustry Association is gratefully acknowledged.

References

Battersby, A. R. (1967). Biosynthesis of the indole and colchicum alkaloids. *Pure and Applied Chemistry*, *14*(1), 117–136.

Battersby, A. R., Westcott, N. D., Glusenkamp, K.-H., & Tiezte, L. F. (1981). Untersuchungen zur Biogenese der Indolalkaloide. Synthese und verfuetterung radioaktiv markierter Hydroxyloganin-Derivate. *Chemische Berichte*, *114*(10), 3439–3447.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254.

Bolwell, G. P., Bozak, K., & Zimmerlin, A. (1994). Plant cytochrome P450. *Phytochemistry*, *37*(6), 1491–1506.

Cordell, G. A. (1974). The biosynthesis of indole alkaloids. *Lloydia*, *37*(2), 219–298.

Dagnino, D., Schripsema, J., & Verpoorte, R. (1995). Terpenoid indole alkaloid biosynthesis and enzyme activities in two cell lines of *Tabernaemontana Divaricata*. *Phytochemistry*, *39*(2), 341–349.

Estabrook, R. W., Cooper, D. Y., & Rosenthal, O. (1963). The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. *Biochemische Zeitschrift*, *338*, 741–755.

Fujita, M., Oba, K., & Uritani, I. (1982). Properties of a mixed function oxygenase catalyzing ipomeamarone 15-hydroxylation in microsomes from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. *Plant Physiology*, *70*(2), 573–578.

Halkier, B. A. (1996). Catalytic reactivities and structure/function relationships of cytochrome P450 enzymes. *Phytochemistry*, *43*(1), 1–21.

Hakamatsuka, T., Hashim, M. F., Ebizuka, Y., & Sankawa, U. (1991). P-450-Dependent oxidative rearrangement in isoflavone biosynthesis: reconstitution of P-450 and NADPH : P-450 reductase. *Tetrahedron*, *47*(31), 5969–5978.

Hakamatsuka, T., & Sankawa, U. (1997). In K. Ogura, & U. Sankawa, *Dynamic aspects of natural products chemistry* (p. 25). Tokyo: Kodansha/Scientific.

Inoue, K., Takeda, Y., Tanahashi, T., & Inouye, H. (1981). On the possibility of the intermediacy of 10-hydroxyloganin in the biosynthesis of secologanin. *Chemical Pharmaceutical Bulletin*, *29*(4), 981–990.

Inoue, K., Tanahashi, T., Inouye, H., Kuwajima, H., & Takaishi, K. (1989). Intermediacy of 6-hydroxyloganin in the ring cleavage course of loganin to secologanin. *Phytochemistry*, *28*(11), 2971–2979.

Kochs, G., & Grisebach, H. (1987). Induction and characterization of a NADPH-dependent flavone synthase from cell cultures of soybean. *Zeitschrift für Naturforschung*, *42c*(4), 343–348.

Lamberts, S. W. J., Bons, E. G., Bruining, H. A., & de Jong, F. H. (1987). Differential effects of the imidazole derivatives etomidate, ketoconazole and miconazole and of metyrapone on the secretion of cortisol and its precursors by human adrenocortical cells. *The Journal of Pharmacology and Experimental Therapeutics*, *240*(1), 259–264.

Mann, J., Davidson, R. S., Hobbs, J. B., Banthorpe, D. V., & Harborne, J. B. (1994). In *Natural products: their chemistry and biological significance* (p. 423). Harlow: Longman Scientific and Technical.

Murashige, T., & Skoog, F. (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473–497.

Rossi, M. (1983). Structural studies of metyrapone: a potent inhibitor of cytochrome P-450. *Journal of Medicinal Chemistry*, *26*(9), 1246–1252.

Saunders, B. C., Holmes-Siedle, A. G., & Stark, B. D. (1964). *Peroxidase*. London: Butterworth.

Sono, M., Roach, M. P., Coulter, E. D., & Dawson, J. H. (1996). Heme-containing oxygenases. *Chemical Review*, *96*(7), 2841–2887.

Tanahashi, T., Nagakura, N., Inouye, H., & Zenk, M. H. (1984). Radioimmunoassay for the determination of loganin and the biotransformation of loganin to secologanin by plant cell cultures. *Phytochemistry*, *23*(9), 1917–1922.

Wendorff, H., & Matern, U. (1986). Differential response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. *European Journal of Biochemistry*, *161*(2), 391–398.

Yamamoto, H., Katano, N., Ooi, A., & Inoue, K. (1999). Transformation of loganin and 7-deoxyloganin into secologanin by *Lonicera japonica* cell suspension cultures. *Phytochemistry*, *50*(2), 417–422.