Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2217–2221

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Development of Plasmin and Plasma Kallikrein Selective Inhibitors and their Effect on M1 (Melanoma) and HT29 Cell Lines

Yoshio Okada,[a,b,*] Yuko Tsuda,[a,b] Keiko Wanaka,[a] Mayako Tada,[a,c] Utako Okamoto,[c] Shosuke Okamoto,[c] Akiko Hijikata-Okunomiya,[d] Gyorgyi Bokonyi,[e] Bela Szende[e] and Gyorgy Keri[e]

[a]*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe 651-2180, Japan*
[b]*High Technology Research Center, Kobe Gakuin University, Kobe 651-2180, Japan*
[c]*Kobe Research Projects on Thrombosis and Haemostasis, Kobe 655-0033, Japan*
[d]*Faculty of Health Sciences, Kobe University School of Medicine, Kobe 654-0142, Japan*
[e]*Semmelweis University of Medicine, Budapest, H-1088, Hungary*

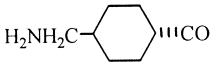
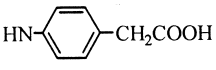
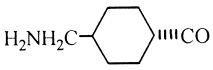
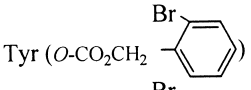
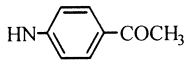
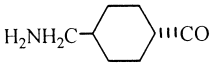
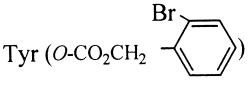
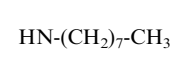
Received 9 June 2000; accepted 29 July 2000

Abstract—*trans*-4-Aminomethylcyclohexanecarbonyl-Tyr(*O*-Pic)-octylamide (YO-2) inhibited plasmin (PL) selectively, while *trans*-4-aminomethylcyclohexanecarbonyl-Phe-4-carboxymethylanilide (YO-1) inhibited plasma kallikrein (PK). YO-2 induced apoptosis of M1 (melanoma) cell line and HT29 colon carcinoma cells during 24 h through activation of caspase-3, while YO-1 did not affect either cell line even during 48 h.

Previously, we reported the development of active center-directed inhibitors of plasmin (PL) and of plasma kallikrein (PK) and studies on the structure–inhibitory activity relationship.[1] Our inhibitors consist of three parts, P1, P1′ and P2′[2] and their structure–inhibitory activity relationship is summarized in Table 1.

As shown in Table 1, compound I (YO-1)[3] inhibits PK specifically, compound II (YO-4)[1] inhibits both PK and PL and compound III (YO-3)[4] inhibits PL more potently than PK. Thus, substitution of position P1′ and/or P2′ with various compounds produces different inhibitory profiles. The mode of interaction of the above inhibitors

Table 1. IC_{50} values (μM) of compounds **I–III** for PL and PK

Compound	P1	P1′	P2′	PL[a]	PK
				S-2251	S-2302
I		Phe		630	1.3
II				0.23	0.37
III				0.80	16

[a]PL: plasmin; PK: plasma kallikrein; S-2251: D-Val-Leu-Lys-pNA; S-2302: D-Pro-Phe-Arg-pNA.

*Corresponding author. Tel.: +81-78-974-1551; fax: +81-78-974-5689; e-mail: okada@pharm.kobegakuin.ac.jp

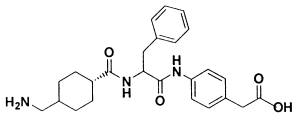
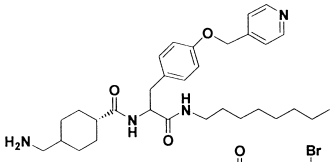
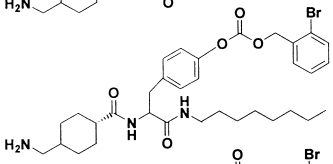
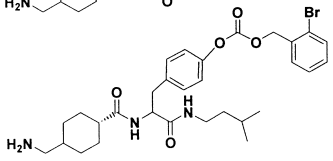
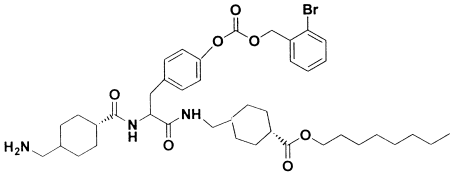
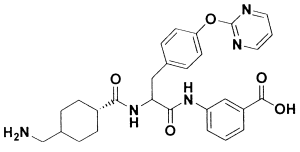
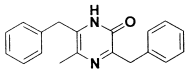
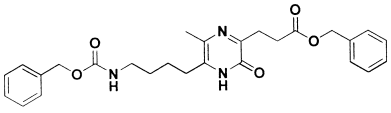
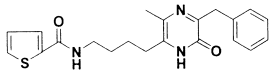
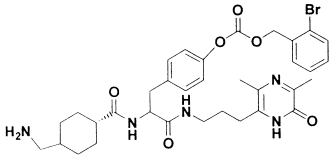
0960-894X/00/$ - see front matter
PII: S0960-894X(00)00431-5

with enzymes was studied by X-ray analysis using *trans*-4-aminomethylcyclohexanecarbonyl(Tra)-Phe-4-carboxymethylanilide (compound I, YO-1)[3] and trypsin.[5]

From the results, it was revealed that amino function of *trans*-4-aminomethylcyclohexanecarbonyl moiety (P1) interacts with Asp^{189} of trypsin. It was also reported that plasmin selective inhibitor, 4-aminomethylbenzoyl-Tyr (*O*-Pic)-hexylamide inhibited the increase of tumor cells in sarcoma 180 bearing mice.[6] Based on this information, our studies were directed to further development of PL and PK selective inhibitors by modifying P1, P1′ and/or P2′ position and examination of their effect on apoptosis of tumor cell lines to develop novel anti-tumor drugs.

As summarized in Table 2, each moiety of YO-1 (P1, P1′ and P2′ in Table 1) was modified to afford compounds (YO-2–YO-10) and their inhibitory activity against var-

Table 2. Inhibitory activity (IC_{50}: μM) of YO-1–YO-10 against various enzymes

Compound	Chemical structure	PL[a]		PK	UK	TH	
		S-2251	Fn	S-2302	S-2444	S-2238	Fg
YO-1		620	350	1.3	350	>1000	>1000
YO-2		0.53	0.36	30	5.3	>400	>100
YO-3		0.80	0.23	16	>50	>50	>25
YO-4		0.46	0.056	2.1	260	70	>100
YO-5		2.5	0.56	45	>50	>50	>10
YO-6		32	29	0.71	650	>1000	>1000
YO-7		—	—	—	—	—	—
YO-8		—	—	—	—	—	—
YO-9		—	—	—	—	—	—
YO-10		0.74	0.39	36	54	490	>250

[a]PL: plasmin; PK: plasma kallikrein; UK: urokinase; TH: thrombin; Fn: fibrin; Fg: fibrinogen; S-2251: D-Val-Leu-Lys-pNA; S-2302: D-Pro-Phe-Arg-pNA; S-2444: Glp-Gly-Arg-pNA; S-2238: D-Phe-Pip-Arg-pNA; —: not detecable.

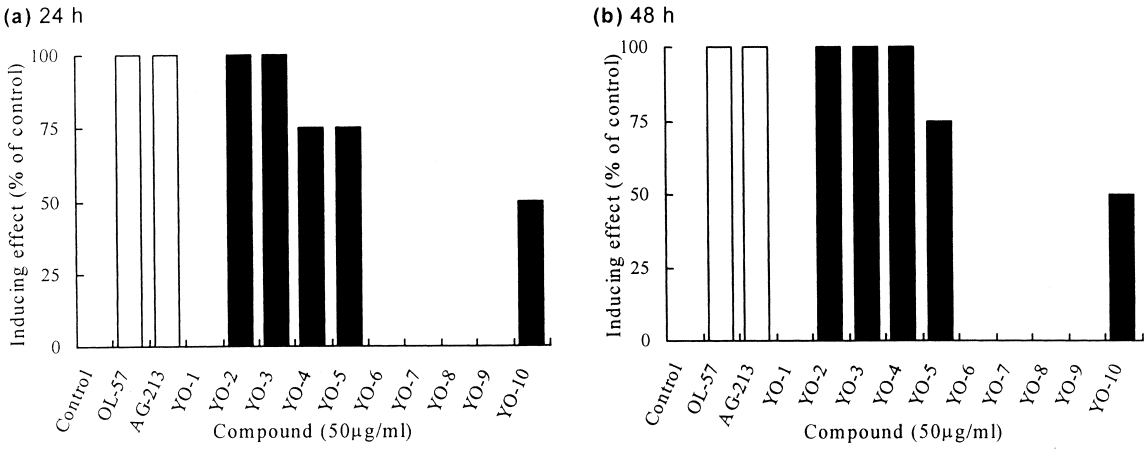


Figure 1. Apoptosis inducing effect of 'YO' compounds on M1 (melanoma) cell line.

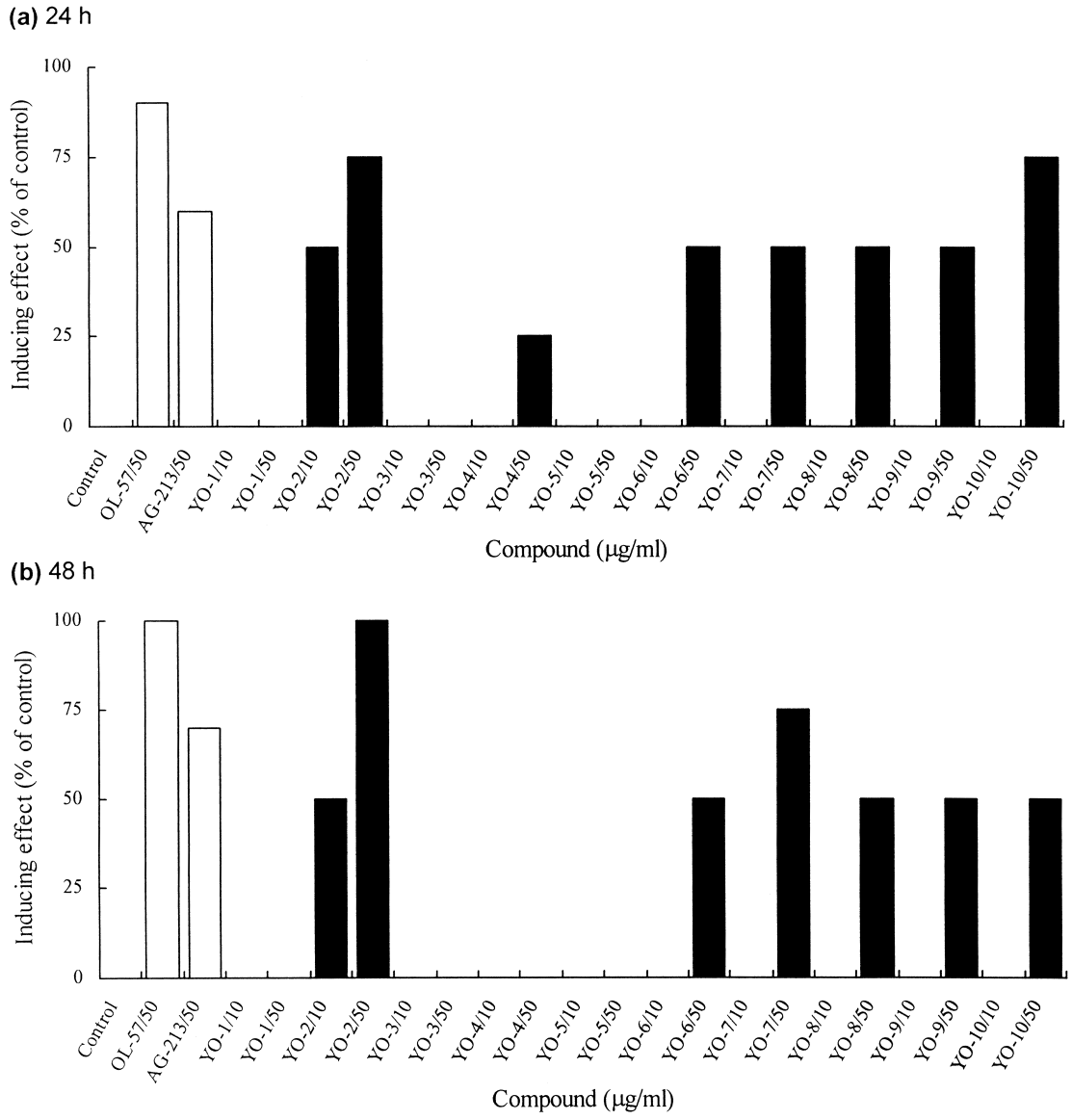


Figure 2. Apoptosis inducing effect of 'YO' compounds on HT29 colon carcinoma cell line.

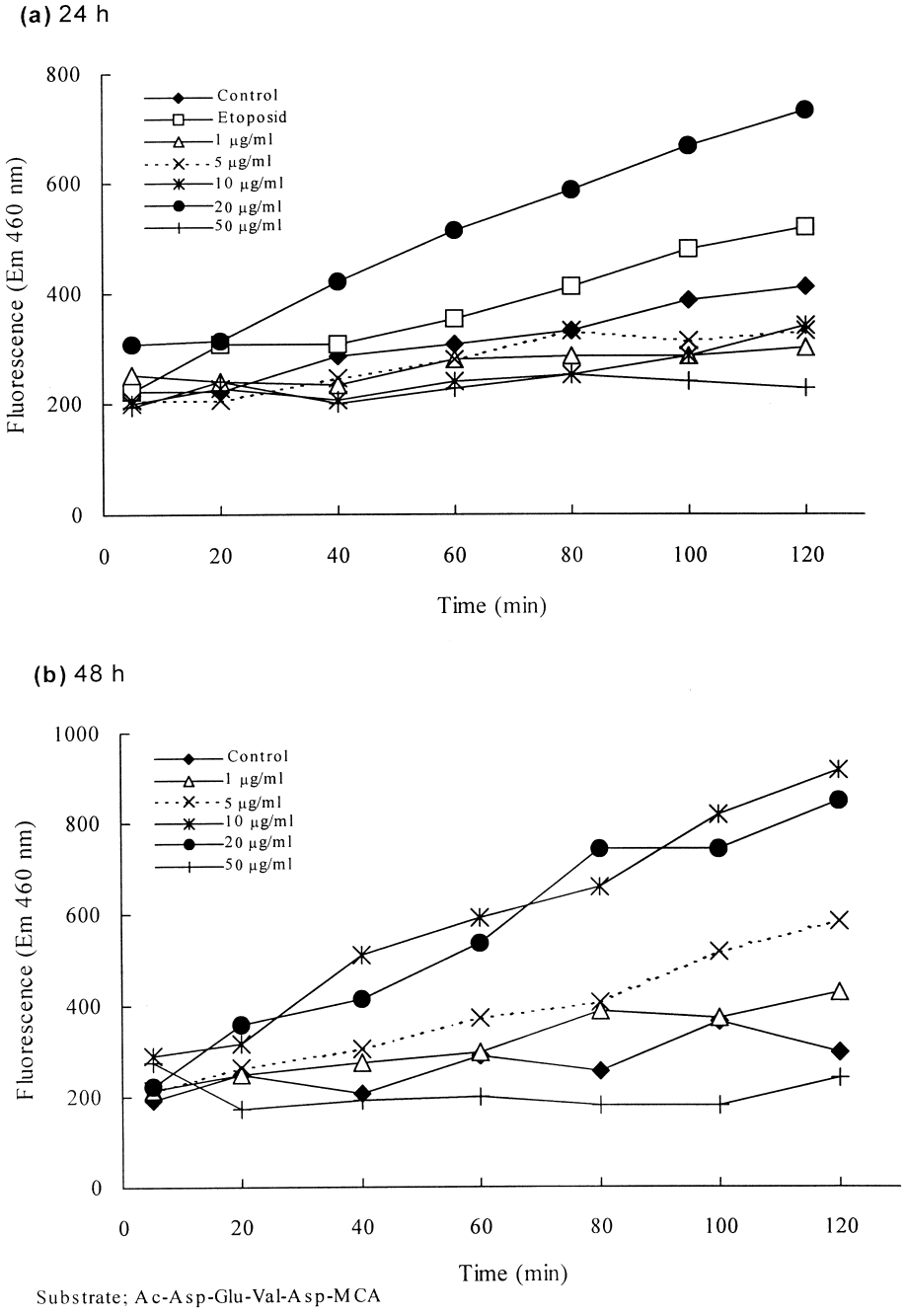


Figure 3. Effect on YO-2 on caspase-3 activity in HT29 colon carcinoma cells.

ious enzymes was examined by the method described previously.[1] Substitution of positions P1′ and P2′ of YO-1 afforded YO-3, YO-4 and YO-5.[4] YO-3 and YO-5 exhibited inhibitory activity against PL, while YO-4 inhibited both PL and PK. YO-2 was derived from YO-3 by substitution at position P1′ and exhibited inhibitory activity against PL and urokinase. YO-6 was derived from YO-1 by substitution at P1′ and P2′ positions to produce potent PK selective inhibitor. YO-7,[7] YO-8[8] and YO-9 do not contain *trans*-4-aminomethylcyclohexanecarbonyl moiety (P1) and do not affect enzymatic activity so far examined. YO-10, derived from YO-3 by substitution at the P2′ position, exhibited PL selective inhibition.

Next, the effect of compounds (YO-1–YO-10) on apoptosis of M1 (melanoma) cells and HT29 colon carcinoma cells was examined. HT29 is a colon carcinoma cell line. The ATCC number is HTB38 and the cell line was obtained from NCI, Bethesda, MC, USA. The M1 melanoma cell line was established in our institute by Ladanyi et al.[9] Treatment of M1 (melanoma) cells or HT29 colon carcinoma cells with YO-compounds was performed in plates containing 24-wells and cells were plated on glass covers. The applied concentrations were 10 and 50 μg/mL and the duration of the treatments was 24 and 48 h. Morphological examination of cells is still the best method to detect apoptosis. Therefore, for the

evaluation of apoptotic events, cells were stained with Haematoxiline-Eosine dyes and counted under light microscope applying the criteria described by Wyllie.[10] The whole sections were observed. As positive controls, OL-57: 6,7-dimethoxy-4-(3′-bromoanilino)quinazoline and AG-213: 3-(3′,4′-dihydrooxyphenyl)-2-cyano-2-propenthioamide were employed.[11]

Their effects on apoptosis of M1 (melanoma) cell lines during 24 h and 48 h are shown in Figure 1(a) and (b), respectively. YO-2 and YO-3, which are selective PL inhibitors, induced significant apoptosis of M1 cell lines during 24 h as OL-57 and AG-213. YO-4, YO-5 and YO-10, which are PL inhibitors, induced strong apoptosis of M1 cell lines during 24 h and YO-4 induced significant apoptosis of M1 cell lines during 48 h. YO-1 and YO-6, which are PK selective inhibitors, did not induce apoptosis of M1 cell lines even during 48 h. YO-7, YO-8 and YO-9, which are not enzyme inhibitors, did not affect apoptosis of M1 cell lines. These results exhibit that PL selective inhibitors are able to induce apoptosis of M1 cell lines; however, PK selective inhibitors do not induce apoptosis of M1 cell lines.

The effect of YO compounds (10 and 50 μg/mL) on apoptosis of HT29 colon carcinoma cells was examined during 24 and 48 h and the results are shown in Figure 2(a) and (b), respectively. YO-1 did not induce apoptosis. YO-2 induced apoptosis potently at 10 μg/mL even during 24 h and at 50 μg/mL exhibited significant apoptosis of HT29 colon carcinoma cells during 48 h as OL-57 (50 μg/mL). YO-3, YO-4 and YO-5 did not induce apoptosis of HT29 colon carcinoma cells even at 50 μg/mL and during 48 h but many large cells with numerous nuclei were observed in the cultures. This is not fragmentation of DNA and may be cell fusion, but not apoptosis. YO-6, which is a PK selective inhibitor, induced apoptosis of HT29 colon carcinoma cells with numerous nuclei in the reaction medium. YO-7, YO-8 and YO-9 induced apoptosis of HT29 colon carcinoma cells, but at the same time numerous mitotic figures were also seen in the cell cultures. YO-10 induced apoptosis of HT29 colon carcinoma cells with numerous mitotic cells in the reaction medium. M1 is a melanoma cell line and HT29 is a colon carcinoma cells and these two tumors may react with reagents differently. However, from the above results, it was revealed that PL selective inhibitors tend to induce apoptosis of tumor cell lines, while PK selective inhibitors do not affect apoptosis. Of them, YO-2 induced apoptosis of both M1 cell lines and HT29 colon carcinoma cells significantly. It did not affect the body weight of mice over a 61 day protocol (data not shown), indicating that YO-2 exhibited no discernible side effect.

In summary, a PL inhibitor could induce apoptosis of tumor cell lines, although the relationship between PL inhibitor and induction of apoptosis is not yet known.

In order to study the mechanism by which YO-2 induces apoptosis in tumor cell lines, the effect of YO-2 on the caspase 3 activity was examined on HT29 colon carcinoma cells after incubation of the cells for 24 or 48 h with YO-2 according to the procedure described previously[12] and the results are shown in Figure 3(a) and (b). 10 μg/mL and 20 μg/mL of YO-2 most effectively activated caspase-3, although 1 μg/mL and 50 μg/mL of YO-2 did not affect caspase-3 activity. 50 μg/mL of YO-2 causes total destruction of cells, therefore no active cell function, like caspase-3 activation can be expected. Thus, lower doses were needed to show caspase-3 activity. It is well known that activation of caspases is critical for the induction of apoptosis.[13] Therefore, it will be deduced that YO-2 induced apoptosis through caspase-3 activation. The relationship between PL inhibitory activity and caspase-3 activation is now under investigation. In vivo anti-tumor activity will be also examined.

References

1. Teno, N.; Wanaka, K.; Okada, Y.; Taguchi, H.; Okamoto, U.; Hijikata-Okunomiya, A.; Okamoto, S. *Chem. Pharm. Bull.* **1993**, *41*, 1079.
2. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
3. Okada, Y.; Tsuda, Y.; Wanaka, K.; Hijikata-Okunomiya, A.; Okamoto, U.; Okamoto, S. *Biopolymers* **1999**, *51*, 41.
4. Okada, Y.; Matsumoto, Y.; Tsuda, Y.; Tada, M.; Wanaka, K.; Hijikata-Okunomiya, A.; Okamoto, S. *Chem. Pharm. Bull.* **2000**, *48*, 184.
5. Nakamura, M.; Tomoo, K.; Ishida, T.; Taguchi, H.; Tsuda, Y.; Okada, Y.; Okunomiya, A.; Wanaka, K.; Okamoto, S. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 583.
6. Wanaka, K.; Okamoto, S.; Horie, N.; Hijikata-Okunomiya, A.; Okamoto, U.; Naito, T.; Ohno, T.; Bohgaki, M.; Tsuda, Y.; Okada, Y. *Thrombosis Res.* **1996**, *82*, 79.
7. Yokoi, T.; Taguchi, H.; Nishiyama, Y.; Igarashi, K.; Kasuya, F.; Okada, Y. *J. Chem. Res. (S)* **1997**, 10.
8. Okada, Y.; Fukumizu, A.; Takahashi, M.; Yamazaki, J.; Yokoi, T.; Tsuda, Y.; Bryant, S. D.; Lazarus, L. H. *Tetrahedron* **1999**, *55*, 14391.
9. Ladanyi, A.; Timar, J.; Paku, S.; Molnar, G.; Lapis, K. *Int. J. Cancer* **1990**, *46*, 456.
10. Wyllie, A. H. *J. Pathol.* **1987**, *153*, 313.
11. Keri, Gy.; Erchegyi, J.; Horvath, A.; Mezo, I.; Idei, M.; Vantus, T.; Balogh, A.; Vadasz, Zs.; Boekoenyi, Gy.; Seprodi, J.; Teplan, I.; Csuka, O.; Tejeda, M.; Gaal, D.; Szegedi, Zs.; Szende, B.; Roze, C.; Kalthoff, H.; Ullrich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12513.
12. Gurtu, V.; Kain, S. R.; Zhang, G. *Anal. Biochem.* **1997**, *251*, 98.
13. Basu, A.; Akkaraju, G. R. *Biochemistry* **1999**, *38*, 4245.