Amino Acids and Peptides. XXXI. Preparation of Analogs of the Laminin-Related Peptide YIGSR and Their Inhibitory Effect on Experimental Metastasis

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Analogs of a partial sequence peptide of laminin, *i.e.*, Tyr–Ile–Gly–Ser–Arg (YIGSR) analogs and Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg (CDPGYIGSR) analogs, were prepared by the solid-phase method and their inhibitory effects on experimental metastasis of B16–BL6 melanoma cells were examined. YIGSR analogs in which Ile was replaced by other hydrophobic amino acids (Met, Leu, Phe) were inhibitory. Cys-containing analogs of YIGSR were also prepared, but were less active than the parent peptide, YIGSR. Among them, CYIGSR was easily oxidized to form a disulfide bond. A Cys-containing YIGSR analog cyclized through a disulfide bond, cyclo(CYIGSRC)G, was prepared. The disulfide bond formation was performed on the resin by the silyl chloride–sulfoxide method and by the iodine oxidation method. The yield of the silyl chloride–sulfoxide method was much better than that of the iodine oxidation method.

Key words laminin; disulfide bond formation; antimetastatic effect; experimental metastasis; peptide synthesis; metastasis

Laminin,[1] a cell adhesion protein consisting of three peptide chains (A, B1, B2), promotes the adhesion and growth of epithelial and tumor cells. Iwamoto *et al.*[2] found that two partial sequence peptides of laminin, Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg (CDPGYIGSR) and Tyr–Ile–Gly–Ser–Arg (YIGSR), inhibited experimental metastasis of tumor cells in mice. We have reported that the antimetastatic effect of YCGSR is more potent than that of YIGSR.[3]

In order to explore the structure–activity relationship, we planned to replace Ile of YIGSR with other hydrophobic amino acids, such as Leu, Met and Phe. The peptides were prepared by the solid-phase method on *p*-methylbenzhydrylamine resin with a *tert*-butyloxycarbonyl (Boc) group as the α-amino-protecting group. The following groups were used as protecting groups for the side chains of amino acids; benzyl group for the hydroxyl groups of Tyr and Ser, tosyl group (Tos) for the guanidino group of Arg. Coupling reactions were performed by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method.[4] The final deprotection was performed by HF treatment[5] and the desired materials were purified by HPLC. The antimetastatic effects of the synthetic peptides were examined in mice.

B16–BL6 melanoma cells (1×10^5 cells/0.2 ml) were intravenously injected into C57BL/6 mice and then YIGSR and YIGSR analogs were i.v. administered to the mice within 1 min after tumor cell-inoculation. Mice were killed 2 weeks later and tumor colonies in the lungs were counted. Metastatic colonies were observed in all mice injected with B16–BL6 melanoma cells. YIGSR at a dose of 1 mg/mouse inhibited the experimental metastasis of B16–BL6 melanoma cells *in vivo*. As shown in Fig. 1, all the synthetic peptides examined exhibited the inhibitory effect. Among the synthetic analogs, YMGSR exhibited the most potent effect. Thus, Ile in YIGSR was not essential for the antimetastatic effect, and can be replaced with various other hydrophobic amino acids such as Met, Phe and Leu. The incubation of B16–BL6 melanoma cells with YIGSR or YIGSR analogs at 3 mg/ml for 1 h showed no evidence of cytotoxicity, so we speculate that the observed antimetastatic effects of YIGSR and its analogs in this study were due to inhibition of the adhesion of B16 melanoma cells to laminin.

Next, Cys-containing YIGSR analogs were prepared. Iwamoto *et al.*[2] reported that the antimetastatic effect of CDPGYIGSR is more potent than that of YIGSR. We speculated that potent inhibitory effect of CDPGYIGSR might be related to the high reactivity of the thiol group in the peptide. As described above, YCGSR[3] was more potent than YIGSR. Thus, we prepared Cys-containing YIGSR analogs (CYIGSR, CGYIGSR, CPGYIGSR, CDPGYIGSR) and compared their antimetastatic effects with those of YIGSR and CDPGYIGSR. All peptides were prepared by the solid-phase method in the

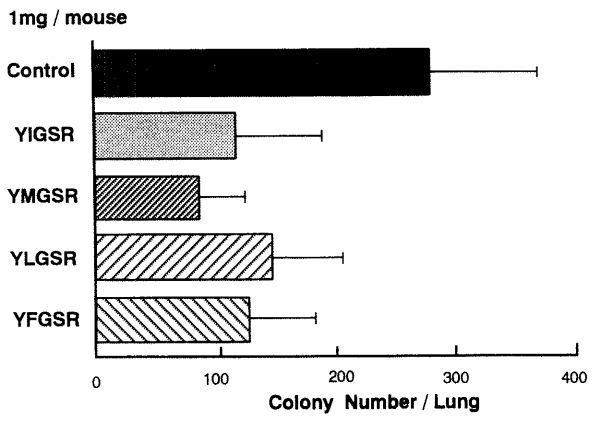

Fig. 1. Inhibitory Effect of YIGSR Analogs on Experimental Metastasis of B16–BL6 Melanoma

B16–BL6 cells and synthetic peptides were injected separately into five mice per group. Control mice received the same amount of cells without the synthetic peptide. Lung tumor colonies were counted 2 weeks later. Bars represent standard error of the mean.

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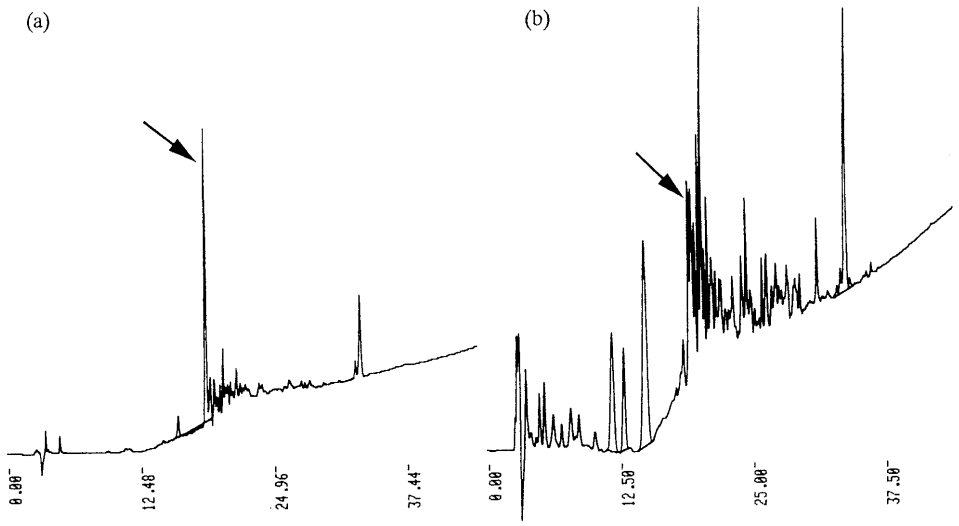

Fig. 2. HPLC Profiles of YIGSR Analogs Cyclized by the Silyl Chloride–Sulfoxide Method and by Iodine Oxidation

(a) Silyl chloride–sulfoxide method. (b) Iodine oxidation. The arrow indicates the peak corresponding to the desired cyclic peptide. Column,Cosmosil 5C 18-AR (20 × 250 mm). Flow rate, 10 ml/min. Eluent, A) 0.1% TFA/water; B) 0.1% TFA/CH_3CN. Gradient, A/B: 90/10→50/50 (0→40 min).

same way as described above. The thiol group of Cys was protected with a *p*-methylbenzyl group and all protecting groups were finally removed by HF treatment. The desired peptides were purified by RP-HPLC. The thiol group of CYIGSR was highly reactive, and CYIGSR readily formed a dimer through disulfide bond formation. After purification by preparative HPLC, CYIGSR was examined by analytical HPLC. A peak corresponding to the dimer of CYIGSR formed through a disulfide bond was detected in addition to the peak of CYIGSR. The whole sample was purified again by HPLC and the purified sample was examined by analytical HPLC. The peak of the dimer was detected again. When an aqueous solution of CYIGSR was stirred overnight and examined by analytical HPLC, CYIGSR was almost completely converted to its dimer. Other Cys-containing peptides were more stable. After purification by preparative HPLC, they showed a single peak corresponding to the desired material by analytical HPLC.

The cyclic, disulfide-bonded YIGSR analog cyclo-(CYIGSRC)G, was also prepared by the solid-phase method. An acetamidomethyl (Acm) group was used as a protecting group for the cysteine thiol group. Akaji *et al.*[6] reported the silyl chloride–sulfoxide method for disulfide formation of Cys(Acm)-containing peptides and they were successful in forming a disulfide bond without damage to Trp and Tyr. In the previous paper,[7] we reported intra-molecular disulfide formation of Cys–Arg–Gly– Asp–Cys on the solid support. Here, we examined intra-molecular disulfide bond formation on the solid support by using these two methods. It is well-known that Tyr is iodinated by iodine treatment. However, we expected that the disulfide-forming reaction by iodine on the resin might be possible, if the phenyl rings in *p*-methylbenzhydrylamine resin (a polystyrene type resin) act as a scavenger to prevent the iodination reaction. Boc–Cys(Acm)–Tyr(Cl_2–Bzl)–Ile–Gly–Ser(Bzl)–Arg-(Tos)–Cys(Acm)–Gly–methylbenzhydrylamine resin was prepared. Since the resin may influence the disulfide-formation reaction, *e.g.*, through steric hindrance, the *C*-terminal Gly was incorporated as a spacer between the resin and Cys(Acm). First, we treated Boc–Cys(Acm)–Tyr(Cl_2–Bzl)–Ile–Gly–Ser(Bzl)–Arg(Tos)–Cys(Acm)–Gly–resin with trichloromethylsilane and diphenyl sulfoxide in 50% trifluoroacetic acid (TFA)/dichloromethane (DCM) for 30 min. The resulting peptide-resin was treated with HF to remove all protecting groups and to cleave the peptide from the resin, followed by purification by HPLC. The Boc–Cys(Acm)–Tyr(Cl_2–Bzl)–Ile–Gly–Ser-(Bzl)–Arg(Tos)–Cys(Acm)–Gly–resin was also treated with iodine in DCM/MeOH for 30 min. The resulting peptide–resin was treated with HF and the product was purified by HPLC. The preparative HPLC profiles of the desired peptide [cyclo(CYIGSRC)G] prepared by the two methods are shown in Fig. 2. The product prepared by the silyl chloride–sulfoxide method gave the main peak corresponding to the desired material, but the product prepared by the iodine oxidation method gave many peaks, with no main peak. Overall yields of the desired material based on the starting resin were 4% by the silyl chloride–sulfoxide method and 0.4% by the iodine oxidation method. The silyl chloride–sulfoxide method developed by Akaji *et al.* is thus superior for disulfide formation of the Tyr-containing peptide.

Cyclization by the solution method was also done. The Boc–Cys(Acm)–Tyr(Cl_2–Bzl)–Ile–Gly–Ser(Bzl)–Arg-(Tos)–Cys(Acm)–Gly–resin was treated with HF to give the Acm derivative, H–Cys(Acm)–Tyr–Ile–Gly–Ser–Arg–Cys(Acm)–Gly–NH_2 in 22% yield based on the starting resin. The Acm derivative was treated with iodine in aqueous acetic acid at 0 °C and the product was purified by HPLC. The yield of iodine oxidation was as high as 67%. Iodination of Tyr might be slow at 0 °C in an acidic aqueous medium. Iodine oxidation of a Tyr-containing peptide in an acidic aqueous medium at low temperature might be possible without serious iodination on Tyr. In the previous paper,[7] we reported that the disulfide bond formation of a fibronectin-related peptide (Cys–Arg–Gly–Asp–Cys) on the resin was superior to that in solution. This was not the case here. However we examined the

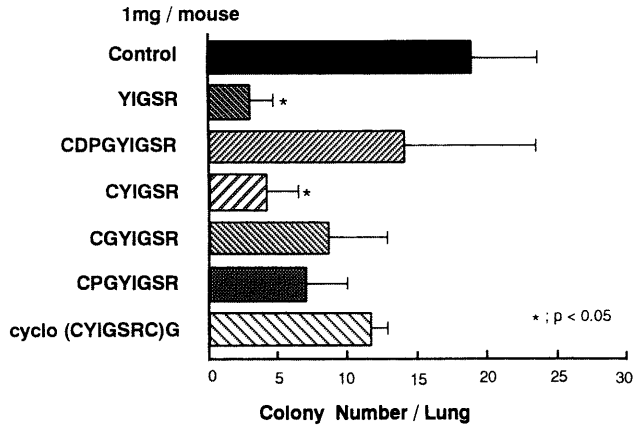

Fig. 3. Inhibitory Effect of Cys-Containing Analogs on Experimental Metastasis of B16-BL6 Melanoma

The experiment was carried out as described in Fig. 1. One mg of each peptide was injected into mice.

disulfide bond formation of Tyr-containing peptide by iodine oxidation on the resin in an organic solvent, and by the solution method in an acidic aqueous medium. The solvent effect on the iodination reaction of Tyr will be studied in future.

The antimetastatic effect of the Cys-containing YIGSR analogs was examined and results are shown in Fig. 3. As mentioned above, CYIGSR is easily oxidized to its dimer, therefore antimetastatic effect of CYIGSR shown in Fig. 3 might be that of a mixture of CYIGSR and its dimer. The antimetastatic effect of YIGSR was more potent than that of CDPGYIGSR. This is inconsistent with the finding by Iwamoto *et al.*,[2] who reported that CDPGYIGSR was more potent than YIGSR. The reason is not clear, might be related to a difference between the assay methods used. Iwamoto *et al.*[2] injected a mixture of the peptide and tumor cells, but we injected the peptide and the cells separately. Many factors may influence *in vivo* metastasis assay.

In conclusion, Ile in YIGSR can be replaced with other hydrophobic amino acids (such as Met, Leu and Phe) without loss of the activity. The synthetic Cys-containing YIGSR analogs were less active than the parent peptide, YIGSR. The cyclic analog through the disulfide bond was also active, but its effect was less than that of YIGSR. The silyl chloride-sulfoxide method was superior to the iodine oxidation method for the disulfide bond formation of the Cys(Acm) peptide performed on the resin.

Experimental

p-Methylbenzhydrylamine resin (amino content, 0.68 meq./g) was purchased from Watanabe Chemical Industries, Ltd. Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h. Amino acid compositions of acid hydrolysates were determined with a Kyowa K-252SN amino acid analyzer. RP-HPLC was conducted with a Waters 600 on a YMC Pack ODS-M80 column or a Cosmosil 5C 18-AR column using gradient systems of CH_3CN/H_2O containing 0.1% TFA. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer.

General Procedure for Peptide Synthesis by the Solid Phase Method Peptides were prepared by a manual method. The synthetic protocol for solid-phase peptide synthesis is shown below. Reactions were checked by mean of the ninhydrin test.[9]

Step	Reagents	Reaction time	
1	N-methylmorpholine/ dichloromethane (DCM)	10 min	×2
2	DCM	3 min	×3
3	Boc-amino acid (2 eq) in DMF 1 M DCC/ dimethylformamide (DMF) (2eq), 1 M HOBt/DMF (2eq)	120 min	
4	50 % MeOH/DCM	5 min	×3
5	DCM	2 min	1
6	50% TFA/DCM, anisole	2 min	1
		40 min	1
7	DCM	3 min	×4

Final Deprotection The synthetic peptide resin was treated with 5% anisole/HF at 0 °C for 1 h. After removal of HF, the residue was washed repeatedly with ether and extracted with 5% AcOH, followed by lyophilization. The product was purified by HPLC. The purified peptide was converted to its hydrochloride by repeated lyophilization from HCl-containing water. Yields were calculated from the amino content of the resin used.

H-Tyr-Leu-Gly-Ser-Arg-NH_2 (YLGSR): Yield 27%, Amino acid ratios in an acid hydrolysate: Tyr 1.02, Leu 1.14, Gly 1.00, Ser 0.93, Arg 1.05 (average recovery 80%). $[\alpha]_D^{28}$ −14.7° (c=1.0, H_2O), FAB-MS m/z: 594 (M+1).

H-Tyr-Met-Gly-Ser-Arg-NH_2 (YMGSR): Yield 11%. Amino acid ratios in an acid hydrolysate: Tyr 1.06, Met 0.83, Gly 1.00, Ser 0.90, Arg 1.05 (average recovery 86%). $[\alpha]_D^{26}$ −8.7° (c=1.0, H_2O), FAB-MS m/z: 612 (M+1).

H-Tyr-Phe-Gly-Ser-Arg-NH_2 (YFGSR): Yield 3%. Amino acid ratios in an acid hydrolysate: Tyr 0.80, Phe 0.83, Gly 1.00, Ser 0.92, Arg 1.12 (average recovery 86%). $[\alpha]_D^{28}$ −6.6° (c=1.0, H_2O), FAB-MS m/z: 628 (M+1).

H-Cys-Tyr-Ile-Gly-Ser-Arg-NH_2 (CYIGSR): Yield 9%. Amino acid ratios in an acid hydrolysate: Cys 0.91, Tyr 1.06, Ile 0.94, Gly 1.00, Ser 0.90, Arg 1.01 (average recovery 81%), $[\alpha]_D^{28}$ −11.4° (c=1.0, MeOH), FAB-MS m/z: 697 $(M+1)^+$.

H-Cys-Gly-Tyr-Ile-Gly-Ser-Arg-NH_2 (CGYIGSR): Yield 18%. Amino acid ratios in an acid hydrolysate: Cys 0.93, Tyr 1.00, Ile 0.93, Gly 2.00, Ser 0.91, Arg 1.04 (average recovery 80%), $[\alpha]_D^{26}$ −8.3° (c=1.0, MeOH), FAB-MS m/z: 754 $(M+1)^+$.

H-Cys-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH_2 (CPGYIGSR): Yield 15%. Amino acid ratios in an acid hydrolysate: Cys 0.80, Pro 0.99, Tyr 0.92, Ile 0.98, Gly 2.00, Ser 0.98, Arg 1.02 (average recovery 77%), $[\alpha]_D^{28}$ −16.5° (c=1.0, MeOH), FAB-MS m/z: 851 $(M+1)^+$.

H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH_2 (CDPGYIGSR): Yield 15%. Amino acid ratios in an acid hydrolysate: Cys 0.70, Asp 0.99, Pro 1.12, Tyr 0.96, Ile 0.98, Gly 2.00, Ser 0.97, Arg 1.09 (average recovery 75%), $[\alpha]_D^{29}$ −33.9° (c=1,0, MeOH), FAB-MS m/z: 966 $(M+1)^+$.

Cyclo(H-Cys-Tyr-Ile-Gly-Ser-Arg-Cys-Gly-NH_2) [Cyclo(CYIGSRC)G]. A) Cyclization by Chloride-Sulfoxide System Boc-Cys(Acm)-Tyr(Cl_2-Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-Cys(Acm)-Gly-resin (1 g) was swollen with dichloromethane (DCM) and was suspended in 50% TFA/DCM (10 ml) containing diphenylsulfoxide (450 mg, 4.6 mmol), trichloromethylsilane (2.7 ml, 46 mmol) and anisole (2.5 ml). The mixture was stirred for 30 min at room temperature and the resin was washed successively with 50% TFA/DCM, DCM and DMF. The resin was dried and was treated with HF, followed by HPLC. Yield 20 mg (4%). Amino acid ratios in an acid hydrolysate: Cys 1.61, Tyr 0.73, Ile 0.97, Gly 2.00, Ser 0.86, Arg 0.93 (average recovery 93%), $[\alpha]_D^{29}$ +7.6° (c=1.0, H_2O), FAB-MS m/z: 855 $(M+1)^+$.

B) Cyclization by Iodine Oxidation. a) Cyclization on the Resin Boc-Cys(Acm)-Tyr(Cl_2-Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-Cys(Acm)-Gly-resin (0.5 g) was swollen with DCM. Iodine (29 mg, 0.46 mmol) and anisole (1 ml) were dissolved in MeOH (10 ml) and the solution was added to the swollen resin. The mixture was stirred for 30 min at room temperature and the resin was washed successively with DCM/MeOH (1/1), DCM and DMF. The resin was dried and treated with HF. The product was extracted with 10% CH_3CN/H_2O and purified by HPLC. Yield 1 mg (0.4%). Amino acid ratios in an acid hydrolysate: Cys 1.50, Tyr 0.75, Ile 0.89, Gly 2.00, Ser 0.87, Arg 0.95 (average recovery 81%). The product was identical by HPLC with the sample prepared by the

silyl chloride–sulfoxide method.

b) Cyclization by the Solution Method Boc–Cys(Acm)–Tyr(Cl_2–Bzl)–Ile–Gly–Ser(Bzl)–Arg(Tos)–Cys(Acm)–Gly–resin (0.6 g) was treated with HF and the product (H–Cys(Acm)–Tyr–Ile–Gly–Ser–Arg–Cys(Acm)–Gly–NH_2) was purified by HPLC. Yield 74 mg (22%). The product (51 mg, 0.04 mmol) was dissolved in 20% AcOH (20 ml). An iodine solution (59 mg/10 ml 80% AcOH) was added dropwise to the solution at 0 °C and the whole was stirred for 2 h at 0 °C. The reaction mixture was washed with ether 3 times and lyophilized. The product was purified by HPLC. Yield 30 mg (67%, calculated from Acm derivative). Amino acid ratios in an acid hydrolysate: Cys 1.69, Tyr 0.79, Ile 0.85, Gly 2.00, Ser 0.92, Arg 0.96 (average recovery 84%). $[\alpha]_D^{19}$ +11.8° (c=1.0, H_2O), FAB-MS m/z: 855 $(M+1)^+$.

Metastasis Assay The metastasis-inhibitory effect of synthetic peptides was examined as reported.[8] Briefly, B16–BL6 melanoma cells (1×10^5/0.1 ml) and a synthetic peptide (1 mg/0.1 ml/mouse) were intravenously injected separately into C57BL/6 mice. The mice were killed at 14 d after tumor inoculation, and the lungs were removed. The number of surface melanoma colonies on the lungs was counted under a stereoscopic microscope.

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References

1) *a*) Timple R., Rohde H., *J. Biol. Chem.*, **254**, 9933—9937 (1979); *b*) Odermatt E., Engel E., *J. Mol. Biol.*, **150**, 97—120 (1981); *c*) Kleinman H. K., Cannon F. B., Laurie G. W., Hassel J. R., Aumailley M., Terranova V. P., Martin G. R., DuBois-Dalcq M., *J. Cell. Biochem.*, **27**, 317—325 (1985).
2) Iwamoto Y., Robey F. A., Graf J., Sasaki M., Kleinman H. K., Yamada Y., Martin G. R., *Science*, **238**, 1132—1134 (1987).
3) Kawasaki K., Namikawa M., Murakami T., Mizuta T., Iwai Y., Hama T., Mayumi T., *Biochem. Biophys. Res. Commun.*, **174**, 1159—1162 (1991).
4) König W., Geiger R., *Chem. Ber.*, **103**, 788—798, 2024—2033 (1970).
5) Sakakibara S., Shomonishi Y., Kisida Y., Okada M., Sugihara H., *Bull. Chem. Soc. Jpn.*, **40**, 2164—2167 (1967).
6) Akaji K., Tatsumi T., Yoshida M., Kimura T., Fujiwara Y., Kiso Y., *J. Am. Chem. Soc.*, **114**, 4137—4143 (1992).
7) Kakiuchi M., Izuno Y., Maeda M., Ueda K., Fujiwara K., Kunitada S., Kawasaki K., *Chem. Pharm. Bull.*, **44**, 1107—1110 (1996).
8) *a*) Kawasaki K., Namikawa M., Yamashiro Y., Hama T., Mayumi T., *Chem. Pharm. Bull.*, **39**, 3373—3375 (1991); *b*) Kawasaki K., Namikawa M., Yamashiro Y., Iwai Y., Hama T., Tsutsumi Y., Yamamoto S., Nakagawa S., Mayumi T., *ibid.*, **43**, 2133—2138 (1995).
9) Kaiser E., Colescotto R. L., Bossinger C. D., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).