Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 28 (2004) 185–190
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

A screening of phage displayed peptides for the recognition of fullerene (C60)

Yasutaka Morita [a], Takuya Ohsugi [a], Yoshihiro Iwasa [b], Eiichi Tamiya [a,*]

[a] *School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292, Japan*
[b] *Institute for Materials Research, Tohoku University, 2-1-1 Katahira, Aobaku, Sendai 980-8577, Japan*

Received 9 October 2003; received in revised form 15 January 2004; accepted 24 January 2004

Abstract

A novel approach to develop a peptide, that can recognize fullerene (C60) is described for affinity selection of phage displayed peptides from a combinatorial peptide library. Biopanning was performed using cyclic 7-mer peptide library against C60 films deposited on silicon (Si) substrate, and eluted phages with organic solvent. The phage, that recognized C60 films deposited on Si substrate, were obtained from biopanning. The nucleotides of the phage, coding a cyclic 7-mer peptide, were sequenced by standard method. Seventeen kinds of peptide displayed phages were obtained. One kind of peptide (peptide No. 4) displayed phage recognized the C60 films deposited on Si substrate. Peptide No. 4 displayed no affinity towards the Si substrate. The recognition event was monitored by a fluorescent immunoassay. Additionally, peptide No. 4 phage could recognize C60 in powder form, but not the graphite powder. This recognition event in powder form was also observed by a fluorescent immunoassay.

Keywords: Peptide; Peptide library; Phage display; Fullerene; C60

1. Introduction

Fullerene (C60) are made exclusively of carbon atoms, in which condensed hexagons and pentagons are arranged in a spherical form similar to a soccer ball. Several fullerenes (C60, C70 etc.) were detected, the most abundant, C60, has received the most attention in the scientific field [1]. C60 displays a wide range of interesting properties, non-linear optical properties, superconductivity, thus it exhibits a potential use in biological field [2,3]. For the biological applications of C60, studies about its neuroprotective [4], anticancer [5], antiviral [6], protease inhibition [7], DNA photocleavage [8], activities have been reported in the recent years. The biological properties of C60 as an element for peptide recognition has attracted our interest [9–12]. Combination of C60 with functional peptides will provide new application possibilities in medical and environmental areas [13,14].

Phage display peptide library is based on a combinatorial library of random peptide fused to a minor coat protein (pIII) of the filamentous M13 phage. Phage display creates a physical linkage between a displayed selectable function and the DNA encoding that function. This allows rapid identification of peptide ligands for a variety of target molecules by an in vitro selection process [15–17]. Phage display is an effective technique for isolation of the peptides and proteins, that can bind to a specific molecule. In the selection of a phage, not only purified proteins immobilized on an article surface or intact cells, but also chemical molecules [18,19] or a surface have been used as the target [11]. This approach makes it possible to use chemicals in physiological condition as the target, so that phage display is suitable for the development of peptides that bind to a molecular antigen. The peptide isolated from a phage display library could conjugate with a particular dye, which makes it applicable to identification and isolation of specific chemicals.

To the best of our knowledge, this report is the first attempt to develop a peptide, that can recognize fullerene using phage display [20,21]. Features of peptide isolation from a phage display library are discussed in the following sessions.

* Corresponding author. Tel.: +81-761-51-1660; fax: +81-761-51-1665.
E-mail address: tamiya@jaist.ac.jp (E. Tamiya).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.01.018

2. Experimental

2.1. Reagents and solution

C60 (99.9%) was purchased from Matsubo Corporation (Tokyo, Japan). Cyclic 7-mer phage display peptide library kit, Ph.D.-C7C™ was purchased from New England BioLab Inc. (MA, USA). The kit is based on a combinatorial library of random peptide 7-mer fused to a minor coat protein of M13 phage. Under nonreducing conditions, the cysteines will spontaneously form a disulfide cross-link, resulting in phage display of cyclized peptides. The library consists of about 2.7×10^9 independent phage particles. Biotin labeled anti-filamentous fd, F1, M13 phages monoclonal antibody were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Avidin–FITC (fluorescein isothiocyanate) label was purchased from Sigma–Aldrich (MO, USA). Si-(1 0 0) oriented substrates (CZ-N type) were obtained from S.E.H. America Inc. (WA, USA). Other reagents were of analytical grade.

2.2. Preparation of C60 films deposited on Si substrates

Si substrates (1 cm × 1 cm) were washed by acetone, and then the surfaces were made hydrophobic by 5% fluoric acid. For screening, the films of C60 were deposited on Si substrates with 3 Ω resistance at room temperature in a conventional heating evaporator (SVC-700 Turbo-TM) from Sanyu Electron Co., Ltd. (Tokyo, Japan). The film of C60 had 300 nm thickness [17].

For fluorescent immunoassay, the films of C60 were deposited on one part of the Si layer in such a way as to cover the other half by aluminum foil.

2.3. Selection of C60 binding phage by biopanning

The screening of phage bound to films of C60 on Si substrate from phage display library was performed as follows. At first, a 15 μl aliquot of phage display library (1.5×10^{13} pfu/ml) was incubated with Si substrate in 35 mm petri plate to remove the phage bound to Si substrate in 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) buffer solution (TBS), and then incubated for 2 h at 4 °C. The phage, which did not bind to Si substrate, was incubated with C60 films deposited on Si substrate in TBS, and then incubated for 2 h at 4 °C with gentle shaking. C60 films deposited on Si substrate was washed five times with TBS containing 0.1% Tween-20 (TBST), over a period of 2 h. The phage bound to C60 films deposited on Si substrate were eluted with 100% methanol, and immediately transferred to Luria-Bertani's broth (LB) medium for cultivation of *Escherichia coli* (*E. coli*) ER2537. An aliquot of the eluate was retained for titration, and the rest of the eluate was amplified by infecting log phase culture of *E. coli*, and amplified for the next round as indicated above. After incubation for 12 h at 37 °C, the cells were centrifuged. The amplified phage in the supernatant was purified by precipitating with 20% polyethlene glycol (PEG), 2.5 M NaCl. The final phage pellet was resuspended in 200 μl TBS containing 0.02% NaN_2, quantified spectrophotometrically, and used for subsequent rounds of biopanning. The eluted phages after five rounds biopanning were mixed with their *E. coli* and plated on LB Xgal/IPTG plates. Phage plaques were blue in color when plated on media containing Xgal (5-bromo-4-chloro-3-indoyl-β-D-thiogalactoside), and IPTG (isopropyl-β-D-thiogalactoside). Blue/white screening was used to select phage plaques.

2.4. Sequencing of phage DNA

Phages from selected phage plaques were purified by polyethylene glycol/NaCl precipitation. Single-stranded DNA was prepared, and its sequence was determined by a dideoxy chain termination method using a DNA sequencer purchased from Applied Biosystems (CA, USA).

2.5. Binding assay of phage peptides to C60 films on Si substrate

A bare Si substrate without C60 films was used as a control against non-specific binding of phase peptide directly on to Si coated surface. C60 films and Si substrate were exposed to 50 μl of the screened phage in TBS for 1 h at 4 °C, and then rinsed five times with TBS to remove the unbound phage. This substrate was introduced to 30 μl of an anti-phage-biotin conjugated antibody (1:900 in TBS) for 30 min, and then rinsed five times with TBS to remove the unconjugated antibody. 10 μl of avidin–FITC label (1:900 in TBS) was applied to the biotin conjugated phage through a biotin–avidin interaction. The surface was exposed to the FITC label for 30 min, and then rinsed several times with TBS to remove the unconjugated avidin–FITC label. The color intensity of Si surface was observed by a fluorescence microscope (MZFLIII, Leica Microsystems, Inc., IL, USA) (excitation filter: 425 ± 60 nm, absorption filter: 480 nm). The surface image was coped with scientific image processing system, IPlab systems (Scanalytics, Inc., VA, USA) for numerical analysis.

Unselected phage display peptide library was used for the unbound phage to C60 as a control against the screened phage. Binding assays were performed by following the same method described above.

2.6. Binding assay of phage peptides to C60 or graphite powder in solution

C60 or graphite powder (1 mg) was mixed with the screened phage in TBS for 1 h, and then rinsed five times with TBS. This powder was introduced to an anti-phage-biotin conjugated antibody for 30 min, and then rinsed five times with TBS. Ten microliter of avidin–FITC label was attached to the biotin conjugated phage through a biotin–avidin interaction, the conjugate was exposed to

the FITC label for 30 min, and then rinsed five times with TBS. The color intensity of these powders was measured as in the method above. The fluorescent intensity of C60 with that of graphite powder was compared.

2.7. Synthesis of FITC labeled No. 4 peptide

For the determination of binding property of synthetic peptide No. 4, the peptide was synthesized by solid phase synthesis using peptide synthesizer (model: PSSM-8, Shimadzu, Kyoto, Japan) [22]. The manual of this synthesizer was followed for the peptide synthesis. Hundred micromole of synthesized peptide was dissolved in 20 mM ammonium acetate buffer (pH 8.0) and naturally oxidized by stirring at 4 °C for 1 day to cyclize the peptide by cysteins. After the reaction, the peptide was freeze-dried. The cyclized peptide was separated on TSK gel ODS-80 Ts column (4.6 mm × 150 mm; Tosoh, Tokyo, Japan) in high performance liquid chromatography (HPLC) equipped with a model CCPM-II multi pump, a model UV8020 ultraviolet (UV) detector, a model FS8020 fluorescence detector, and a model SC-8020 system controller (Tosoh). The peptides were eluted at room temperature with 0.1% (v/v) trifluoroacetic acid (TFA) dissolved in 50% (v/v) acetonitrile in H_2O. The eluted peptide fraction was collected under UV at an absorbance of 222 nm. The cyclic peptide (No. 4 peptide) was determined by matrix-assisted laser desorption ionization time of fright (MALDI-TOF) mass spectrometric analysis (Voyager RP, Applied Biosystems). α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used for matrix substance of MALDI-TOF.

For labeling of FITC to N-terminal of No. 4 peptide, 100 mg FITC was mixed with 74 mg No. 4 peptide (500 ml) in 0.05 M boric acid buffer (pH 9.2) by stirring at 4 °C for 12 h [23,24]. After the reaction, the peptide was freeze-dried for 2 days. FITC labeled No. 4 peptide was separated in HPLC as same condition described above. The eluted peptide fraction was collected under fluorescence detector. Excitation wavelength was fixed at 494 nm for FITC, and emission spectrum was measured at 519 nm. FITC labeled No. 4 peptide was determined by MALDI-TOF mass spectrometric analysis. α-CHCA was used for matrix substance of MALDI-TOF.

3. Results and discussion

3.1. Selection of phage displayed peptides that bind to C60

In seeking peptides for the recognition of C60, a cyclic 7-mer phage display peptide library was employed for affinity selection of phages capable of binding to C60. In the screening steps, 100% methanol was used to elute the phage bound to C60. The eluted phage was confirmed to have an ability of infection to *E. coli* by titration step. After five rounds of biopanning, the binding of phage clones to the target C60 was screened by blue/white color plaques using Xgal and IPTG. Seventeen positive clones were selected. Seven kinds of DNA sequencing revealed the amino acid sequences shown in Table 1. There were no major consensus among the 17 peptide sequences. The peptide No. 4 (amino acid sequence: CNMSTVGRC) displayed phage was selected as the one with the highest affinity C60 among 7 phages in Table 1 by preliminary binding assay. In the preliminary binding assay, the peptide No. 2, No. 4, and No. 7 displayed phages were pick up, and compared their binding efficiencies by binding assay. The peptide No. 4 displayed phage was apparently strongest binder than No. 2 and No. 7 peptide (data no shown). The peptide No. 4 displayed phage was further characterized.

Table 1
Sequences of peptides derived from the 7-mer phage display peptide library

Code	Amino acid sequence
No. 1	C–S–R–T–P–T–S–Q–C
No. 2	C–S–R–P–N–H–G–T–C
No. 3	C–S–H–M–Q–T–N–L–C
No. 4	C–N–M–S–T–V–G–R–C
No. 5	C–S–N–A–S–V–K–P–C
No. 6	C–L–G–P–N–T–K–L–C
No. 7	C–L–N–P–T–N–S–S–C

Amino acid sequences of peptide inserts were deduced from DNA sequencing of the insert region of the positive phage clones. The cysteins of both end sides spontaneously formed a disulfide cross-link, resulting in phage display of cyclized peptides.

3.2. Binding assay of screened peptide No. 4

The peptide No. 4 displayed phage was used to confirm its binding ability. C60 films and Si substrate were exposed to the peptide No. 4 displayed phage or an unselected display peptide library. The surface-bound under went further binding events involving anti-phage-biotin conjugated antibody and avidin–FITC label. The surface was observed by a fluorescence microscope, and a fluorograph was obtained after integration for 15 s. The surface image was coped with a scientific image processing system for numerical analysis. No significant increase in fluorescence intensity was observed, when an unselected phage display peptide library was incubated with C60 films and Si substrate (Fig. 1A). The reason for the low fluorescent signal was that the library of 2.7×10^9 independent phages containing the ones, that could bind to either Si substrate or C60, was used in the assay. However, strong fluorescence was observed, when the peptide No. 4 displayed phage exposed to C60 films (Fig. 1B). In the case of bare Si substrate with peptide No. 4 displayed phage, the fluorescence was found to be low. These results indicated that the peptide No. 4 displayed phage recognized C60 film, and not bound to Si substrate.

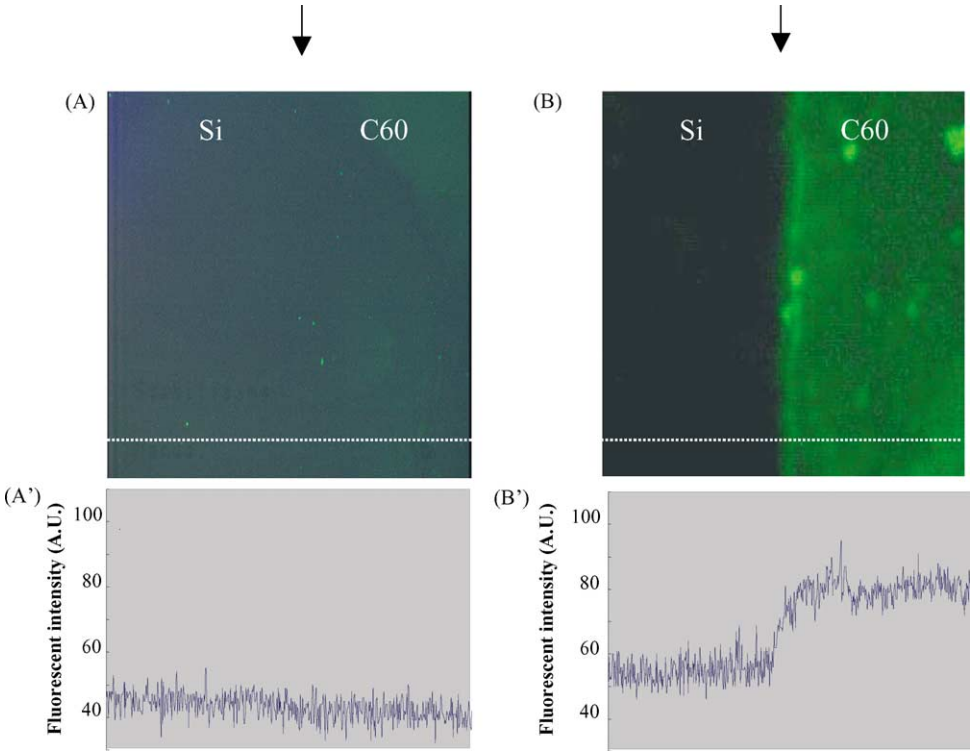

Fig. 1. Binding assay of phage peptide No. 4 to C60 films on Si substrate. Left side of the surface is bare Si substrate, and right side is deposited with C60 films. Arrowheads in (A) and (B) indicates the boundary between Si substrate and C60 films. Phage display peptide library (A) or peptide No. 4 phage (B) was incubated with C60 films and Si substrate. Binding of the phages to C60 films were detected with anti-phage-biotin conjugated antibody and avidin–FITC label through a biotin–avidin interaction. The fluorescence intensity of the respective surface images (A' and B') showed the binding event between peptide No. 4 phage and C60 films.

3.3. Binding specificity of the peptide No. 4 on the allotrope

Binding assay of phage peptides to C60 and graphite was carried out, because graphite is also one of the allotropes of carbon. The graphite powder was used for binding assay, because it did not evaporate on the surface of Si substrate. So, the binding property of peptide No. 4 displayed phage was compared against C60 and graphite powder. The peptide No. 4 displayed phage was incubated with either C60 powder or graphite powder. The bound phage with powder was detected with fluorescence using anti-phage-biotin conjugated antibody and avidin–FITC label. The powder sample was monitored by a fluorescence microscope, and a fluorograph was obtained after integration for 4 s. No fluorescent particles were detected with graphite powder (Fig. 2A). Particles with strong fluorescence were observed with C60 powder (Fig. 2B). These results showed that the peptide No. 4 displayed phage recognized the carbon allotrope between C60 and graphite powder.

Hydrophobic or aromatic amino acids were expected to exist in the peptide, that could bind to C60 [20,21]. In fact, actually, hydrophilic amino acids were found to be more than the hydrophobic ones in the peptide No. 4. Thus, it was concluded that the hydrophobicity was not the key element for binding to C60. Graphite has a tabular crystal structure. On the other hand, C60 has a spherical structure, which is 1.034 nm (i.d.). Furthermore, the putative diameter of cyclic peptide No. 4 is 1.2 nm (data not shown). The reason that peptide No. 4 could preferably bind to C60, but not to graphite, may be that the circular structure of peptide No. 4 encapsulated the spherical C60. The target C60 may be bound to peptide No. 4 with hydrophobic interaction between cystine, methionine, valine, and glycine, and Van der Waals force. Furthermore, the structure of No. 4 peptide may surround the target C60 like the binding pocket of the antibody.

The binding property of synthetic peptide No. 4 was also determined. FITC labeled peptide No. 4 was prepared by solid phase synthesis [9,10]. Under alkaline conditions, the cysteines formed a disulfide cross-link, resulting in cyclized peptides. FITC labeled peptide No. 4 was incubated with C60 film or C60 powder. The phage bound to C60 film or C60 powder was detected with labeled FITC in the peptide. Only a slight fluorescence was observed, when FITC labeled peptide was incubated with C60 films and C60 powder. It means that FITC labeled peptide No. 4 did not recognize C60. Probably, FITC (MW: 389.39) had a significant effect on the structure of peptide No. 4, which was a larger molecule (MW: 950.3). Alternatively, a minor coat protein fused to the displayed peptide, contributed a structural stability to the peptide No. 4 phage.

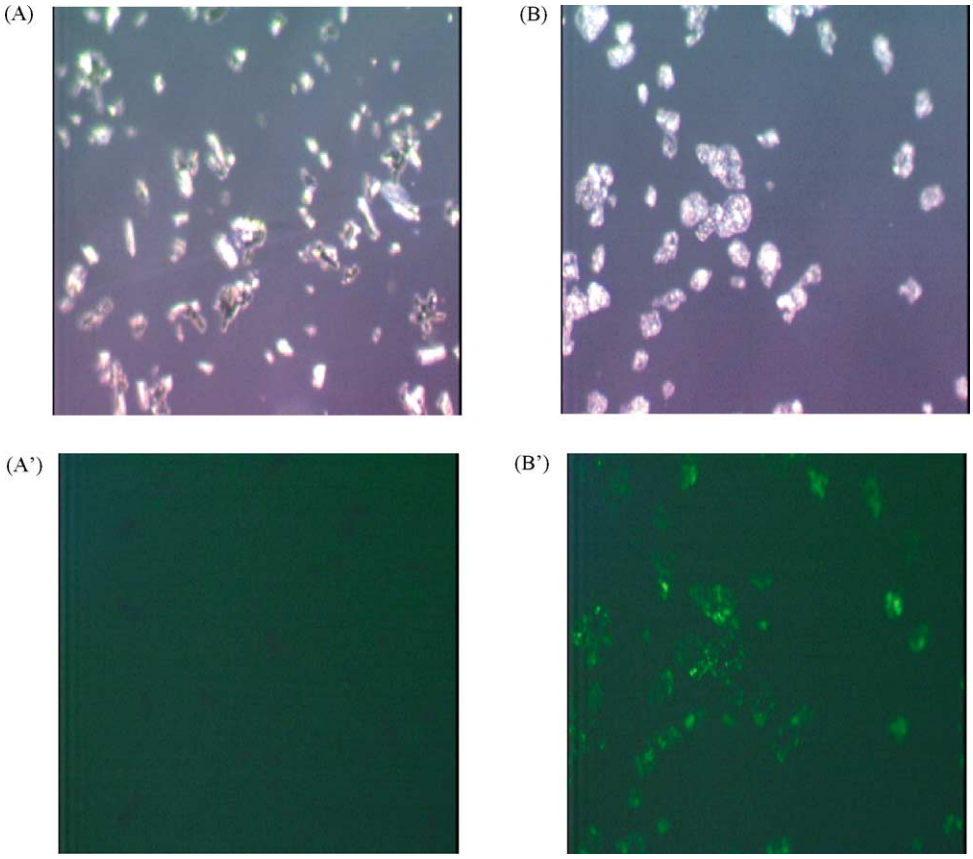

Fig. 2. Binding assay of phage peptides to fullerene powder or graphite powder in solution. (A) and (B) are light field photographs. (A') and (B') are fluorographs. Peptide No. 4 phage was incubated with graphite powder (A, A') or C60 powder (B, B'). Bindings of the phages to C60 were detected with anti-phage-biotin conjugated antibody and avidin–FITC label through a biotin–avidin interaction.

The peptide isolated from a phage display library could conjugate with a particular dye by using avidin–biotin interaction, which makes it applicable to identification and isolation of specific chemicals. In the case of the peptide No. 4 bound to C60, this peptide can be used as a tool for purification of C60, and also as an the immobilized reagent for water soluble C60 in biological samples.

4. Conclusions

In order to reduce unspecific binding of phages to the fullerene, the phage peptide libraries were pre-adsorbed to Si substrate before each selection on the C60. After the five rounds of selection, peptide No. 4 displayed phage (amino acid sequence: CNMSTVGRC) was isolated. Immuno-fluorescence analysis revealed that No. 4 phage selectively bound to C60, but not to Si substrate or graphite powder. The chemically synthesized FITC peptide No. 4 with FITC label did not recognize neither C60 film nor C60 powder. The identified peptide is probable peptide to be a useful tool for purification, an assistant reagent for water soluble C60, or an immobilizing reagent for C60.

Acknowledgements

We are thankful to Pharm. Kagan Kerman for critical reading of the manuscript. This work was partially funded by a Research for Future Program from the Japan Society for Promotion of Science (JSPS).

References

[1] H.W. Kroto, J.R. Heath, S.C. O'Brien, R.F. Curl, R.E. Smalley, Nature 318 (1985) 162.
[2] T.D. Ros, M. Prato, Chem. Commun. (1999) 663.
[3] Y. Yamakoshi, S. Sueyoshi, Bull. Natl. Health Sci. 117 (1999) 50.
[4] L.L. Dugan, D.M. Turetsky, C. Du, D. Lobner, M. Wheeler, C.R. Almli, C.K. Shen, T.Y. Luh, D.W. Choi, T.S. Lin, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 9434.
[5] Y. Tabata, Y. Murakami, Y. Ikeda, Fullerene Sci. Technol. 5 (1997) 989.
[6] F. Käserman, C. Kempf, Antiviral Res. 34 (1997) 65.
[7] A. Wlodawer, M. Miller, M. Jaskólski, B.K. Sathyanarayana, E. Baldwin, I.T. Weber, L.M. Selk, L. Clawson, J. Schneider, S.B.H. Kent, Science 245 (1989) 616.
[8] A.S. Boutorine, H. Tokuyama, M. Takasugi, H. Isobe, E. Nakamura, C. Helene, Angew. Chem. Int. Ed. Engl. 33 (1994) 2464.

[9] Y. Morita, K. Yokoyama, E. Tamiya, in: R. Epton (Ed.), Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Libraries 2000, Mayflowerwide, 2001, p. 325.
[10] Y. Morita, Y. Murakami, K. Yokoyama, E. Tamiya, ACS Symposium Series 830, in: M.R. Marten, T.H. Park, T. Nagamune (Ed.), American Chemical Society, 2002, p. 210.
[11] S.R. Whaley, D.S. English, E.L. Hu, P.F. Barbara, A.M. Belcher, Nature 405 (2000) 665.
[12] B.-X. Chen, S.R. Wilson, M. Das, D.J. Coughlin, B.F. Erlanger, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 10809.
[13] A. Bianco, T.D. Ros, M. Prato, C. Toniolo, J. Peptide Sci. 7 (2001) 208.
[14] F. Pellarini, D. Pantarotto, T.D. Ros, A. Giangaspero, A. Tossi, M. Prato, Org. Lett. 3 (2001) 1845.
[15] J. Zhang, H. Spring, M. Schwab, Cancer Lett. 171 (2001) 153.
[16] N. Venkatesh, Y. Zaltsman, D. Somjen, B. Gayer, E. Boopathi, R. Kasher, T. Kulik, E. Katchalski-Katzir, F. Kohen, Peptides 23 (2002) 573.
[17] V. Capozzi, G. Celentano, G. Perna, G.F. Lorusso, A. Minafra, J. Lumin. 86 (2000) 129.
[18] J. Zhang, H. Spring, M. Schwab, Cancer Lett. 171 (2001) 153.
[19] M. Essler, E. Ruoslahti, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 2252.
[20] B.C. Braden, F.A. Goldbaum, B.X. Chen, A.N. Kirschner, S.R. Wilson, Natl. Acad. Sci. U.S.A. 97 (2000) 12193.
[21] A. Bianco, C. Corvaja, M. Crisma, D.M. Guldi, M. Maggini, E. Sartori, C. Toniolo, Chem. Eur. J. 8 (2002) 1544.
[22] Y. Morita, Y. Murakami, K. Yokoyama, E. Tamiya, in: M.R. Marten, T.H., Park, T. Nagamune (Ed.), ACS Symposium Series 830 Biological systems Engineering, American Chemical Society, Washington, DC, 2002, Chapter 17, p. 210.
[23] M.W. Bromer, S.K. Sheehan, A.W. Berns, E. Arquilla, Biochemistry 6 (1969) 2378.
[24] H. Maeda, N. Ishida, H. Kawauchi, K. Tuzimura, J. Biochem. 65 (1969) 777.