Application of an Automated Synthesis Suite to Parallel Solution-Phase Peptide Synthesis

Noritaka KURODA,*[,a] Taeko HATTORI,[a] Yoko FUJIOKA,[a] David G. CORK,[a] Chieko KITADA,[b] and Tohru SUGAWARA[a]

Discovery Research Laboratories V,[a] Discovery Research Division, Takeda Chemical Industries, Ltd., 17–85 Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532–8686, Japan and Discovery Research Division,[b] Takeda Chemical Industries, Ltd., Wadai-10, Tsukuba, Ibaraki 300–4293, Japan. Received April 4, 2001; accepted June 6, 2001

An in-house developed automated synthesis suite was used to prepare a library of 72 tetrapeptide derivatives, the starting materials for pharmaceutically attractive pentapeptides, employing a convergent strategy. An initial set of 18 dipeptides were synthesized on a large-scale (100—1000 g) using automated synthesis workstations, and then 72 tetrapeptides were synthesized on a medium scale (5—10 g) using an automated system. Each di- or tetrapeptide was prepared in a single operating cycle using a modified methanesulfonic acid method, then a sub-library of 56 pentapeptides were synthesized in parallel, on a small-scale (100 mg—1 g) using a robotic workstation.

Key words tetrapeptide; pentapeptide; automated synthesis apparatus; convergent strategy; modified methanesulfonic acid method

Since the introduction of cross-linked polystyrene beads as supports for peptide synthesis, a technology for which Merrifield[1] received the Nobel prize, the use of heterogeneous polymer supports has expanded into many different regions of organic chemistry, for example, solid-phase combinatorial synthesis. Although synthesis on solid-phase supports has proven to be of much value, the heterogeneous nature of the reaction can cause several limitations, such as nonlinear kinetic behavior, solvation problems, lowered reactivities, unequal distribution and/or access to the polymer-bound reaction site, and the fundamental problems of converting solution-based reaction protocols for use under heterogeneous conditions. To overcome some of these problems, Mutter and Bayer pioneered an approach to peptide synthesis that replaced insoluble cross-linked resins with soluble polymer supports, in a strategy termed solution-phase organic synthesis.[2,3] Janda and his coworkers constructed a pentapeptide library using a soluble, linear homopolymer [polyethylene glycol monomethyl ether (MeO-PEG)] that also served as a terminal protecting group for the compounds, and found high affinity (K_d=7.1 nM) to a specific anti-β-endorphin antibody in a binding-assay.[4]

We have been developing automated synthesis workstations and systems that can handle solution-phase technology,[5] and have applied them to the synthesis of many compound libraries, including some bioactive oligopeptides.[6] For example, in the automated synthesis of a peptide library, we first synthesized 72 L–D–L–D tetrapeptides, described by the general formula *tert*-Butoxycarbonyl-X–D-Ala–Y–D-Ala-OBzl (where X and Y were randomly selected from L-Leu, L-Ser(Bzl), L-Glu(O-cHex), L-Pro, L-Lys(Z), L-Tyr(Bzl), L-Trp, L-Arg(Tos), L-His(Bom)), and then prepared 504 D–L–D–L–D pentapeptides, described by the general formula Boc-Z–X–D-Ala–Y–D-Ala-OBzl (where Z was randomly selected from D-Leu, D-Ser(Bzl), D-Glu(O-cHex), D-Pro, D-Lys(Z), D-Tyr(Bzl), D-Trp, D-Arg(Tos), D-His(Bom), and X and Y were the same as above). Such oligopeptides have the possibility to form β-turn structures in their polypeptide chains, and further derivatization, by deprotection, cyclization or fragment condensation, can lead to pharmaceutically attractive β-turn mimics.

There are two basic strategies, linear and convergent,[7] that can be used for the automated syntheses of compounds consisting of chains of repetitive units. At first, the linear strategy may seem suited to automated repetitive synthesis, since similar reaction procedures can be repeated in sequence, in a fully-automated manner, to complete the process. However, as the number of steps increases, problems are often encountered due to decreased product solubility and increased amounts of contamination by unreacted starting materials, which make it difficult to obtain high yields of pure products. Moreover, if some trouble occurs at any stage, it is necessary to resynthesize or purify from the first step. On the other hand, the convergent strategy is favorable for library construction when the number of units to be joined increases, because the intermediates, such as di- or tetra-peptides, can be isolated and purified before being combined in a variety of different ways.

In our laboratories we have many types of automated synthesis apparatus, applicable to a variety of synthesis scales and reaction conditions. In this paper, we show how they were used as an automation suite to accomplish the library synthesis of 72 tetrapeptide derivatives, and a sub-library of 56 pentapeptides, employing a convergent strategy.

Results and Discussion

The total strategy that was used to construct the tetra- and pentapeptide libraries is shown in Chart 1. Initially, 9 dipeptide benzyl esters were synthesized on a 400 mmol scale and then about half of each of these were converted to dipeptide carboxylic acids, by either saponification or catalytic reduction with ammonium formate as a hydrogen donor. Saponification could be performed on a 200 mmol scale in the 2 l reaction flask (RF) of FUTOSHI,[8] and the heterogeneous catalytic reduction could be carried out on a similar scale by performing multi-batch cycles using ASRA-COL,[9] which has a column shaped flask that can handle solid reagents.

The dipeptide benzyl esters and dipeptide carboxylic acids were then divided into nine fractions, and tetrapeptides were synthesised by condensation of these on a 12 mmol scale,

* To whom correspondence should be addressed. e-mail: Kuroda_Noritaka@takeda.co.jp

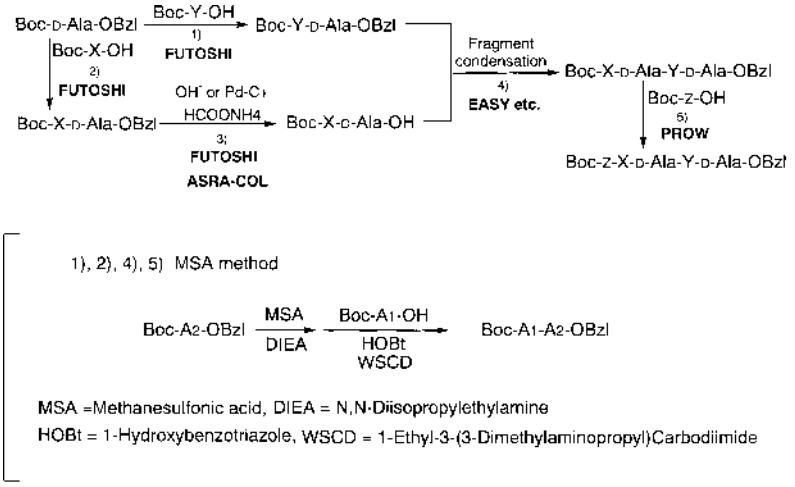

Chart 1. Convergent Strategy for Pentapeptides

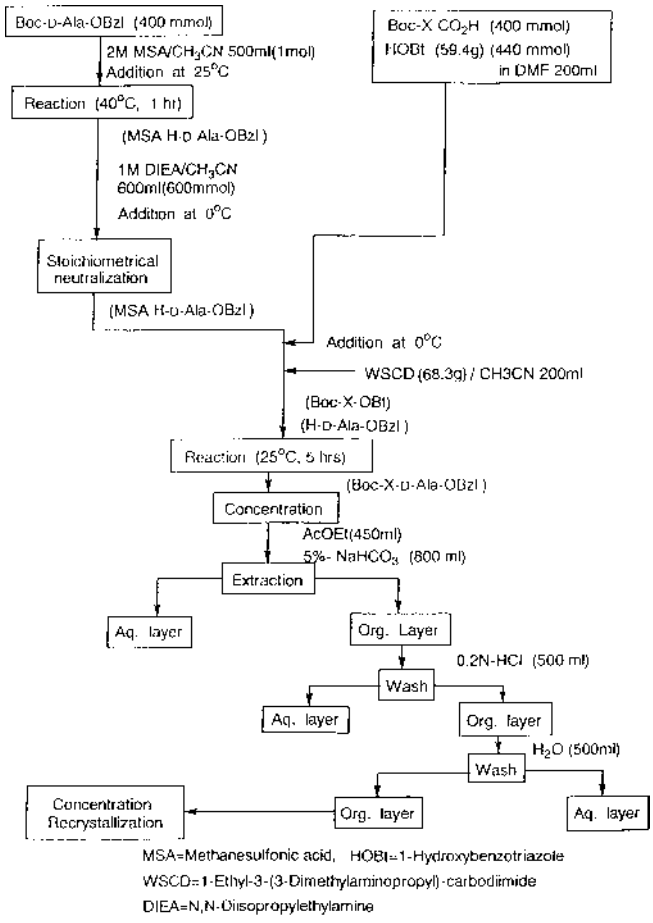

Fig. 1. Flowchart of a Dipeptide Synthesis by the MSA Method

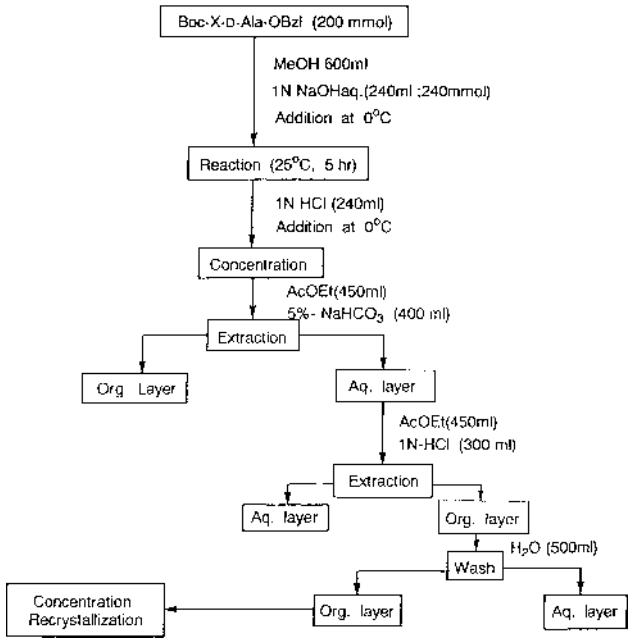

Fig. 2. Folwchart for Saponification of Dipeptide Benzyl Esters

using a modified methanesulfonic acid (MSA) method,[10] in our medium-scale automated synthesis apparatus EASY, TAFT, and ASTRO.[11] A part of some of the tetrapeptides was then derivatized further to prepare a sub-library of pentapeptides, using a robotic workstation, PROW,[12] that can perform up to 60 reactions in parallel.

a) Synthesis of the Starting Dipeptide Derivatives The flowchart for the synthesis of dipeptide benzyl esters by the MSA method is shown in Fig. 1, and the sequence of subroutines used to control the workstation, FUTOSHI, is listed in Table 1. The flowchart for the saponification of the dipeptide benzyl esters, and the related subroutine sequence, is shown in Fig. 2, and Table 2, respectively. However, among these derivatives it was necessary to saponify the benzyl ester Boc-L-Glu(O-cHex)–D-Ala-OBzl by a different deprotection method because the O-cyclohexyl protecting group on the side chain may also be cleaved under alkaline conditions. Thus, we used the heterogeneous catalytic hydrogen transfer reaction with methanol–ammonium formate. Fortunately, our automated system, ASRA-COL, can handle heterogeneous reactions such as catalytic hydrogenation or metal oxidation/reduction, as it is equipped with a shakable column-shaped flask, which is fitted with filters so that the reaction mixture can be easily separated from the catalyst for product isolation.

Table 3 shows the yields and elemental analysis data of the dipeptide benzyl esters and carboxylic acids. It was clear that the dipeptide benzyl esters were obtained in excellent yields (av. 90%), and the dipeptide carboxylic acids were obtained,

Table 1. Subroutine Sequences for a Dipeptide Synthesis

1. START	2. RF-ST-ON	3. RR1-RF	4. RF-LF-UP
5. REA1 (40c, 60 m)	6. RF1-T-ON (0c)	7. RR2-RF	8. MATU
9. SF-RF	10. RR3-RF	11. RF1-T-OF	12. REA1 (25c, 600 m)
13. CON1 (50c, 300 m)	14. RF-LF-DN	15. RS4-RF	16. RS2-RF
17. RF-MIX	18. RF-SF	19. SEP-SR1	20. SR0-SF
21. SR1-RF	22. RS4-RF	23. RF-MIX	24. RF-SF
25. SEP-SR1	26. SR0-SF	27. SF-RF	28. RF-DR
29. SF-RF	30. RF-MIX	31. RF-SF	32. SEP-SR1
33. SF-RF	34. RS1-RF	35. RF-MIX	36. RF-SF
37. SEP-SR1	38. SF-CF	39. RS4-RF	40. RF-MIX
41. RF-SF	42. SF-BUBB	43. SF-CF	44. ALARM
45. SR1-DR	46. WS-RR1	47. WS-RR2	48. WS-RR3
49. RF-MIX	50. RF-SF	51. SF-BUBB	52. SF-SR1
53. SR1-BUBB	54. SR1-RF	55. RF-SF	56. SF-CF
57. WS-RR1	58. WS-RR2	59. WS-RR3	60. RF-MIIX
61. RF-SF	62. SF-BUBB	63. SF-SR0	64. SR0-BUBB
65. SR0-SF	66. SF-RF	67. RF-DR	68. RF-ST-OF
69. RF-LF-UP	70. RF-DRY	71. RF-LF-DN	72. END

Table 2. Subroutine Sequences for a Saponification

1. START	2. RF-ST-ON	3. RF1-LF-UP	4. RF1-T-ON (0c)
5. RR1-RF	6. RF1-T-OF	7. RF1-T-ON (0c)	8. RR3-RF
9. RF1-T-OF	10. CON1 (40c, 600 m)	11. RF1-T-ON (10c)	12. RF1-T-OF
13. MATU	14. SF-RF	15. RS4-RF	16. RF-MIX
17. RF-SF	18. SEP-SR1	19. SF-DR	20. SR1-RF
21. RS2-RF	22. RS3-RF	23. RF-MIX	24. RF-SF
25. SEP-SR1	26. SF-SR0	27. SR1-RF	28. RS3-RF
29. RF-MIX	30. RF-SF	31. SEP-SR1	32. SF-SR0
33. SR1-RF	34. RF-DR	35. SR0-RF	36. RS1-RF
37. RF-MIX	38. RF-T-OF	39. RF-LF-DN	40. RF-SF
41. SEP-SR1	42. SF-CF	43. RS3-RF	44. RF-MIX
45. RF-SF	46. SF-BUBB	47. SF-CF	48. ALARM
49. SR1-DR	50. WS-RR1	51. WS-RR2	52. WS-RR3
53. RF-MIX	54. RF-SF	55. SF-BUBB	56. SF-SR1
57. SR1-BUBB	58. SR1-RF	59. RF-SF	60. SF-CF
61. WS-RR1	62. WS-RR2	63. WS-RR3	64. RF-MIX
65. RF-SF	66. SF-BUBB	67. SF-SR0	68. SR0-BUBB
69. SR0-RF	70. RF-SF	71. SF-CF	72. RF-ST-OF
73. RF-LF-UP	74. RF-DRY	75. RF-LF-DN	76. END

Table 3. Yield and Elemental Analysis Data of Dipeptide Derivatives

$$\text{Boc-Y–D-Ala-OBzl} \xrightarrow[\text{HOBt, WSCD}]{\text{Boc-X–D-Ala-OH}} \text{Boc-X–D-Ala–Y–D-Ala-OBzl}$$

X	Type	Yield (%)	*Anal.*					
			Calcd			Found		
			C	H	N	C	H	N
L-Leu	1	80	64.24	8.22	7.14	64.40	8.22	7.41
L-Ser(Bzl)	1	95	65.77	7.06	6.14	65.57	6.86	6.40
L-Glu(O-cHex)	1	81	63.65	7.81	5.71	63.54	7.75	6.09
L-Pro	1	83	63.81	7.50	7.44	63.55	7.51	7.62
L-Lys(Z)	1	95	64.31	7.26	7.76	64.25	7.06	7.84
L-Tyr(Bzl)	1	98	69.91	6.81	5.26	69.95	6.71	5.48
L-Trp	1	93	67.08	6.71	9.03	67.03	6.62	9.16
L-Arg(Tos)	1	90	57.03	6.67	11.88	56.94	6.69	11.94
L-His(Bom)	1	93	64.91	6.76	10.44	64.63	6.70	10.59
L-Leu	2	78	55.80	8.36	9.29	55.49	8.70	9.18
L-Ser(Bzl)	2	86	59.00	7.15	7.64	58.79	7.42	7.18
L-Glu(O-cHex)	2	71	57.13	7.82	7.01	56.95	7.91	6.90
L-Pro	2	58	54.53	7.74	9.78	54.88	7.94	10.01
L-Lys(Z)	2 (Dicyclohexylamine salt)	90	64.53	8.92	8.85	64.11	9.16	9.08
L-Tyr(Bzl)	2	98	63.84	6.92	6.20	63.57	6.68	6.25[a]
L-Trp	2	97	66.88	8.69	10.06	67.02	9.17	9.63
L-Arg(Tos)	2	99	48.39	6.85	13.44	48.73	6.82	13.53[b]
L-His(Bom)	2	80	56.89	6.94	12.06	56.11	6.91	12.39[b]

a) 0.5 H_2O, *b*) 1.0 H_2O.

either by saponification or catalytic hydrogenation, in moderate yields (av. 75%). Automated catalytic hydrogenation was performed successfully, using a cyclic batch process, with regeneration of the Pd–C catalyst.[13] The catalyst could be used 3—5 times after which it was necessary to change the teflon filters in the flask as they became blocked by the fine particles of the catalyst.

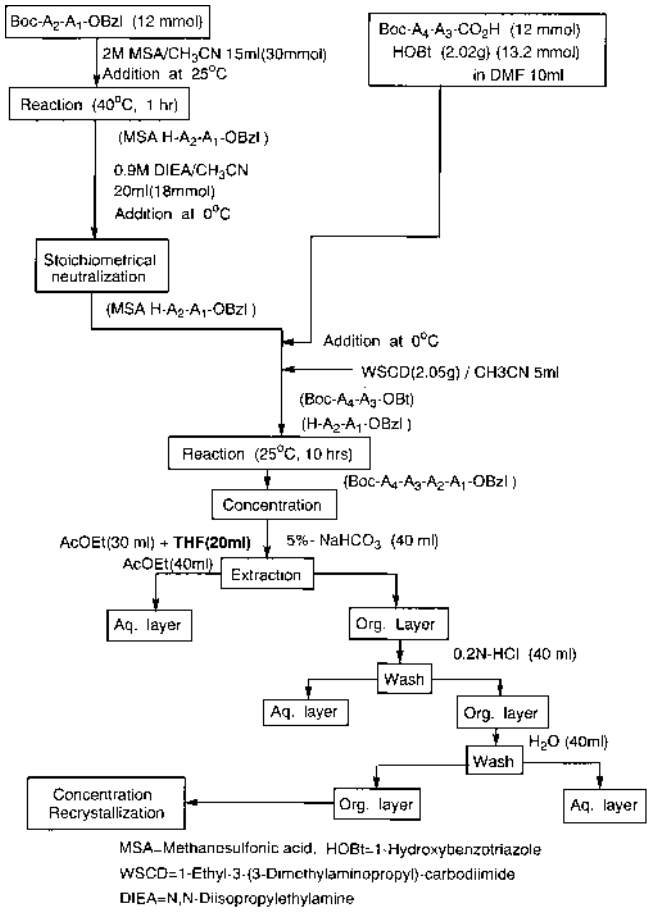

Fig. 3. Flowchart for Tetrapeptide Synthesis by the MSA Method

b) Fragment Condensation of Dipeptides to Tetrapeptides The flowchart for the tetrapeptide synthesis using the MSA method is shown in Fig. 3. For this series, it was necessary to slightly modify the previously-used procedure. For example, the low solubility of tetrapeptides in ethyl acetate caused them to precipitate in the apparatus or prevented them from being extracted into the organic layer, so the solvent was changed to a mixture of tetrahydrofuran and ethyl acetate. Also, the concentration of MSA was increased (from 1 to 2 M) in order to reduce the total solution volume to allow a single batch, 12 mmol-scale synthesis in a 100 ml vessel. The subroutine sequence for the experiment is shown in Table 4, with yields and physical data of the tetrapeptides shown in Table 5.

The starting dipeptides containing histidyl or arginyl groups gave low yields, probably due to the low purity of the starting materials (Boc-L-His(Bom)–D-Ala-OBzl and Boc-L-Arg(Tos)–D-Ala-OBzl). Also, tetrapeptides that included tryptophyl or tyrosyl units, were sometimes precipitated in the apparatus. The degree of racemization for some of the tetrapeptides, randomly sampled from the library, was determined by HPLC analysis, and a value of 4 to 7% was found to be representative of the whole library (see Table 6). Thus the amount of racemization found when using WSCD/HOBt reagents was generally lower than observed for fragment condensation (9.8%).[10b] Since it is not clear whether the D-isomer or L-isomer is going to be bioactive in the first stage of screening, it was considered valid to use the tetrapeptide samples to produce pentapeptides. ^{1}H-NMR data of representative samples are shown in Table 6.

c) Parallel Solution-Phase Synthesis of 56 Pentapeptides A sub-library of 56 pentapeptides, denoted by Boc-Z–L-Pro–D-Ala–Y–D-Ala-OBzl where Z is randomly selected

Table 4. Subroutine Program for a Tetrapeptide Synthesis

1. START	2. RF1-ST-ON	3. RF2-ST-ON	4. RR1-RF1
5. RF1-LF-UP	6. REA1 (40c, 60 m)	7. RF1-T-ON (0c)	8. RR2-RF1
9. MATU	10. RF2-RF1	11. RF6-RF2	12. RF2-MIX
13. RF2-RF1	14. RF2-ST-OF	15. RR3-RF1	16. RF1-T-OF
17. REA1(25c, 600 m)	18. CON1 (40c, 60 m)	19. RF1-LF-DN	20. RS5-RF1
21. RS4-RF1	22. RS2-RF1	23. RF1-MIX	24. BKTETRANK
25. RF1-ST-OF	26. ALARM	27. SR1-DR	28. BKWASHTET
29. BKWASHTET	30. RF1-LF-UP	31. RF2-LF-UP	32. RF1-ST-ON
33. RF2-ST-ON	34. RF1-DRY	35. RF2-DRY	36. RF1-LF-DN
37. RF2-LF-DN	38. RF1-ST-OF	39. RF2-ST-OF	40. END
BKTETRANK			
1. BKSTART	2. RF1-SF	3. SF-BUBB	4. SEP-SR1
5. SF-SR0	6. SR1-SF	7. RS4-RF1	8. RS5-RF1
9. RF1-MIX	10.RF1-SF	11. SF-BUBB	12. SEP-SR1
13. SF-SR0	14. SR1-DR	15. SR0-SF	16. RS3-RF1
17. RF1-SF	18. SF-BUBB	19. SEP-SR1	20. RS1-RF1
21. RF1-SF	22. SF-BUBB	23. SEP-SR1	24. SF-RF3
25. RS4-RF1	26. RS5-RF1	27. RF1-MIX	28. RF1-SF
29. SF-BUBB	30. SF-SR0	31. SR0-BUBB	32. SR0-SF
33. SF-RF3	34. BKEND		
BKWASHTET			
1. BKSTART	2. WS-RR1	3. WS-RR2	4. WS-RR3
5. WS-RF1	6. WS-RF2	7. RF1-BUBB	8. RF2-BUBB
9. RF1-SF	10. SF-BUBB	11. SF-SR1	12. SR1-BUBB
13. SR1-SF	14. SF-SR0	15. SR0-BUBB	16.SR0-DR
17. RF2-RF1	18. RF1-SF	19. SF-RF3	20. BKEND

Table 5. Yield and Physical Data of Tetrapeptides

$$\text{Boc-Y–D-Ala-OBzl} \xrightarrow[\text{HOBt, WSCD}]{\text{Boc-X–D-Ala-OH}} \text{Boc-X–D-Ala–Y–D-Ala-OBzl}$$

X	Y	Yield (%)	*Anal.*					
			Calcd			Found		
			C	H	N	C	H	N
L-Leu	L-Ser(Bzl)	83	63.73	7.55	8.74	63.69	7.32	8.86
L-Leu	L-Glu(O-c-Hex)	78	62.29	8.07	8.30	62.04	7.99	8.31
L-Leu	L-Pro	80	62.12	7.91	9.99	62.05	7.64	10.03
L-Leu	L-Lys(Z)	84	62.88	7.64	9.65	63.01	7.34	9.57
L-Leu	L-Tyr(Bzl)	87	67.02	7.31	7.82	66.67	7.34	7.84
L-Leu	L-Trp	68	63.81	7.34	10.63	63.91	7.35	10.62[a]
L-Leu	L-Arg(Tos)	76	56.76	7.21	12.52	56.90	7.22	12.68[a]
L-Leu	L-His(Bom)	55	62.53	7.32	11.51	62.69	7.14	11.57[a]
L-Ser(Bzl)	L-Leu	70	63.73	7.55	8.74	63.61	7.36	8.86
L-Ser(Bzl)	L-Glu(O-c-Hex)	94	63.40	7.37	7.58	63.35	7.22	7.71
L-Ser(Bzl)	L-Pro	81	62.54	7.16	8.84	62.15	6.82	9.33[b]
L-Ser(Bzl)	L-Lys(Z)	89	63.86	7.02	8.87	63.62	6.98	9.13
L-Ser(Bzl)	L-Tyr(Bzl)	78	67.67	6.71	7.17	67.21	6.60	7.21
L-Ser(Bzl)	L-Trp	77	65.62	6.64	9.81	65.38	6.72	9.67
L-Ser(Bzl)	L-Arg(Tos)	77	58.14	6.66	11.58	58.32	6.25.	
L-Glu(O-c-Hex)	L-Leu	87	62.29	8.07	8.30	62.55	7.98	8.36
L-Glu(O-c-Hex)	L-Ser(Bzl)	77	62.63	7.41	7.49	62.70	7.06	7.55[a]
L-Glu(O-c-Hex)	L-Pro	93	61.15	7.70	8.39	61.25	7.69	8.42[a]
L-Glu(O-c-Hex)	L-Lys(Z)	87	62.68	7.46	8.50	62.54	7.17	8.56
L-Glu(O-c-Hex)	L-Tyr(Bzl)	88	66.32	7.17	6.87	66.18	7.27	6.96
L-Glu(O-c-Hex)	L-Trp	74	64.24	7.14	9.36	64.27	7.05	9.38
L-Glu(O-c-Hex)	L-Arg(Tos)	53	57.85	7.05	11.24	57.55	6.99	11.25
L-Glu(O-c-Hex)	L-His(Bom)	63	62.38	7.18	10.15	62.46	7.06	10.20[a]
L-Pro	L-Leu	76	62.12	7.91	9.99	61.97	7.64	10.02
L-Pro	L-Ser(Bzl)	78	63.44	7.10	8.97	63.36	7.02	8.83
L-Pro	L-Glu(O-c-Hex)	68	61.99	7.65	8.50	62.05	7.55	8.42
L-Pro	L-Lys(Z)	72	62.61	7.24	9.87	62.34	7.27	10.24
L-Pro	L-Tyr(Bzl)	84	66.84	6.90	7.99	66.80	6.76	7.85
L-Pro	L-Trp	72	61.80	7.02	10.60	62.12	6.87	10.61[c]
L-Pro	L-Arg(Tos)	69	57.05	6.78	12.94	56.82	6.57	12.79
L-Pro	L-His(Bom)	59	63.05	6.86	11.92	62.85	6.72	11.83
L-Lys(Z)	L-Leu	90	62.88	7.64	9.65	62.89	7.41	9.82
L-Lys(Z)	L-Ser(Bzl)	91	63.86	7.02	8.87	63.74	6.77	8.93
L-Lys(Z)	L-Glu(O-c-Hex)	61	62.68	7.46	8.50	62.71	7.28	8.50
L-Lys(Z)	L-Pro	85	62.61	7.24	9.87	62.23	7.27	9.80
L-Lys(Z)	L-Tyr(Bzl)	92	66.57	6.87	8.09	66.40	6.81	7.94
L-Lys(Z)	L-Trp	74	64.64	6.81	10.52	64.33	7.11	10.57
L-Lys(Z)	L-Arg(Tos)	65	58.55	6.77	12.14	58.48	6.81	12.10
L-Lys(Z)	L-His(Bom)	59	62.22	6.92	11.04	62.16	6.79	10.96[b]
L-Tyr(Bzl)	L-Leu	88	67.02	7.31	7.82	66.76	7.35	7.71
L-Tyr(Bzl)	L-Ser(Bzl)	89	67.67	6.71	7.17	67.61	6.47	7.13
L-Tyr(Bzl)	L-Glu(O-c-Hex)	76	66.32	7.17	6.87	65.99	7.15	6.85
L-Tyr(Bzl)	L-Pro	60	65.99	6.96	7.89	66.07	6.78	7.98[a]
L-Tyr(Bzl)	L-Lys(Z)	94	66.57	6.87	8.09	66.29	6.78	8.17
L-Tyr(Bzl)	L-Trp	91	68.42	6.51	8.87	68.04	6.62	8.52
L-Tyr(Bzl)	L-Arg(Tos)	70	60.56	6.60	10.52	60.85	6.50	10.72[b]
L-Tyr(Bzl)	L-His(Bom)	59	65.59	6.65	9.56	65.61	6.50	9.21[b]
L-Trp	L-Leu	90	64.70	7.29	10.78	64.56	7.20	10.70
L-Trp	L-Ser(Bzl)	84	65.62	6.64	9.81	65.59	6.53	10.00
L-Trp	L-Glu(O-c-Hex)	76	64.24	7.14	9.36	64.00	7.02	9.31
L-Trp	L-Pro	91	63.53	6.90	10.90	63.16	6.86	10.72[a]
L-Trp	L-Lys(Z)	67	63.92	6.86	10.40	63.97	6.91	10.49[a]
L-Trp	L-Tyr(Bzl)	78	66.90	6.61	8.67	67.18	6.41	8.66[b]
L-Trp	L-Arg(Tos)	71	58.32	6.53	12.95	58.25	6.55	12.94[b]
L-Trp	L-His(Bom)	61	64.32	6.53	12.21	64.50	6.40	12.30[a]

Table 5. Continued

X	Y	Yield (%)	*Anal.* Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
L-Arg(Tos)	L-Leu	79	57.42	7.00	12.35	57.84	6.97	12.34
L-Arg(Tos)	L-Ser(Bzl)	61	58.76	6.62	11.70	58.64	6.51	11.75
L-Arg(Tos)	L-Glu(O-c-Hex)	79	57.26	7.09	11.13	57.38	6.83	11.23[a]
L-Arg(Tos)	L-Pro	84	57.05	6.78	12.94	56.87	6.88	12.72
L-Arg(Tos)	L-Lys(Z)	67	57.99	6.81	12.02	58.08	6.71	11.95[a]
L-Arg(Tos)	L-Tyr(Bzl)	73	61.16	6.55	10.62	61.11	6.59	10.90[a]
L-Arg(Tos)	L-Trp	98	59.56	6.43	13.23	59.29	6.44	13.06
L-Arg(Tos)	L-His(Bom)	80	58.87	6.48	13.73	59.29	6.54	13.43
L-His(Bom)	L-Leu	73	63.31	7.27	11.66	63.12	7.27	11.68
L-His(Bom)	L-Ser(Bzl)	77	64.27	6.68	10.71	63.98	6.48	10.83
L-His(Bom)	L-Glu(O-c-Hex)	85	61.71	7.23	10.04	61.48	6.90	9.92[b]
L-His(Bom)	L-Pro	81	62.26	6.92	11.77	62.36	6.77	11.73[a]
L-His(Bom)	L-Lys(Z)	99	63.50	6.84	11.27	63.63	6.66	11.35
L-His(Bom)	L-Tyr(Bzl)	92	66.27	6.60	9.66	66.22	6.63	9.51[a]
L-His(Bom)	L-Trp	74	63.61	6.58	12.08	63.50	6.48	12.00[b]
L-His(Bom)	L-Arg(Tos)	53	57.74	6.57	13.73	57.79	6.53	13.65[b]

a) 0.5 H_2O, *b*) 1.0 H_2O, *c*) 1.5 H_2O.

Table 6. ^{1}H-NMR and Racemization Data of Tetrapeptide Derivatives (Boc-A_4–A_3–A_2–A_1–OBzl)

A_4–A_3–A_2–A_1	Solvent	Chemical shift (200 MHz): δ (ppm)	Racemization value (%)
L-Ser(Bzl)–D-Ala–L-Glu(O-cHex)–D-Ala	$CDCl_3$	1.35 (3H, s), 1.38 (3H, s), 1.43 (9H, s), 1.47—2.52 (14H, m), 3.51—3.92 (2H, m), 4.25—4.48 (1H, m), 4.45—4.80 (3H, m), 5.13 (2H, s), 5.16 (2H, s), 5.45—5.53 (1H, br s), 6.98 (1H, d, J=7.8 Hz), 7.17 (1H, d, J=7.8 Hz), 7.25—7.45 (10H, m)	5.8
L-Tyr(Bzl)–D-Ala–L-His(Bom)–D-Ala	$CDCl_3$	1.11—1.42 (15H, m), 2.84—3.32 (4H, m), 4.12—4.78 (6H, m), 5.01 (2H, s), 5.11 (2H, d, J=6.6 Hz), 5.29 (2H, s), 6.52 (2H, d, J=7.8 Hz), 6.88 (3H, br s), 7.07(2H, d, J=8.8 Hz), 7.27—7.46 (14H, m), 7.49 (1H, s)	6.4
L-Leu–D-Ala–L-Lys(z)–D-Ala	$CDCl_3$	0.90 (6H, d, J=5.8 Hz), 1.35 (3H, s), 1.38 (3H, s), 1.42 (9H, s), 1.48—2.11 (6H, m), 3.18 (2H, d, J=6.0 Hz), 4.01—4.12 (1H, m), 4.34—4.59 (3H, m), 5.06—5.22 (4H, m), 6.80 (1H, br s), 7.06 (1H, br s), 7.28—7.39 (10H, m)	5.9
L-His(Bom)–D-Ala–L-Leu–D-Ala	$CDCl_3$	0.89 (3H, d, J=6.2 Hz), 0.92 (3H, d, J=6.2 Hz), 1.27 (3H, d, J=6.8 Hz), 1.41 (2H, d, J=6.8 Hz), 1.43 (9H, s), 1.43—1.82 (3H, m), 1.98 (2H, s), 3.03—3.20 (2H, m), 4.28—4.61 (3H, m), 4.51 (2H, s), 5.13 (2H, d, J=8.0 Hz), 5.29 (2H, s), 5.60 (1H, d, J=7.8 Hz), 6.81 (1H, d, J=7.0 Hz), 6.88 (1H, br s), 7.09 (1H, d, J=7.0 Hz), 7.27—7.48 (10H, m)	5.7
L-Arg(Tos)–D-Ala–L-Pro–D-Ala	$CDCl_3$	1.35 (3H, s), 1.38 (3H, s), 1.47 (9H, s), 1.47—2.52 (8H, m), 2.39 (3H, s), 2.82—2.98 (1H, m), 3.42—3.72 (2H, m), 3.85—4.34 (3H, m), 4.44 (2H, d, J=7.6 Hz), 5.12 (2H, d, J=10.6 Hz), 6.59—6.80 (3H, m), 7.22 (2H, d, J=8.0 Hz), 7.25—7.98 (3H, m), 7.34 (5H, s) 7.87 (2H, d, J=8.0 Hz)	6.2
L-Lys(z)–D-Ala–L-Arg(Tos)–D-Ala	$CDCl_3$	1.32 (3H, s), 1.37 (3H, s), 1.43 (9H, s), 1.36—1.92 (10H, m), 2.35 (3H, s), 3.08—3.28 (4H, m), 4.40—4.58 (4H, m), 5.06 (2H, s), 5.11 (2H, d, J=6.2 Hz), 5.18—5.29 (1H, br s), 5.58 (1H, d, J=6.2 Hz), 6.49 (2H, br s), 7.18 (2H, d, J=7.8 Hz) 7.20 (2H, d, J=6.2 Hz), 7.32 (12H, s), 7.75 (2H, d, J=6.2Hz)	5.7
L-Pro–D-Ala–L-Trp–D-Ala	$CDCl_3$	1.22 (3H, d, J=7.0 Hz), 1.27 (3H, d, J=7.0 Hz), 1.43 (9H, s), 1.77—2.08 (4H, m), 3.22—3.45 (2H, m), 4.10—4.23 (1H, m), 4.52 (1H, t, J=7.0 Hz), 4.75 (1H, d, J=8.4 Hz), 5.10 (2H, s), 7.03—7.25 (6H, m), 7.27—7.38 (5H, m), 7.61 (1H, d, J=6.8 Hz), 8.06 (1H, br s)	4.7
L-Glu(O–cHex)–D-Ala–L-Tyr(Bzl)–D-Ala	$CDCl_3$	1.27 (3H, d, J=8.4 Hz), 1.34 (3H, d, J=8.4 Hz), 1.42 (9H, s), 1.44—2.12 (4H, m), 2.41 (2H, d, J=7.4 Hz), 3.06 (2H, dd, J=7.8, 10.8 Hz), 4.05—4.20 (1H, m), 4.27 (1H, t, J=7.0 Hz), 4.48—4.74 (3H, m), 5.02 (2H, s), 5.14 (2H, s), 5.43 (1H, d, J=7.8 Hz), 6.77—6.90 (4H, m), 7.10 (2H, d, J=8.4 Hz), 7.29—7.45 (10H, m)	4.9

from D-Leu, D-Ser(Bzl), D-Glu(O-cHex), D-Lys(Z), D-Tyr(Bzl), D-Trp, D-Arg(Tos), D-His(Bom), and Y is selected from L-Leu, L-Ser(Bzl), L-Glu(O-cHex), L-Lys(Z), L-Tyr(Bzl), L-Trp, L-Arg(Tos), L-His(Bom), were synthesized using our robotic workstation, PROW. The flowchart for the parallel synthesis is shown in Fig. 4. The workstation PROW, which is primarily designed for optimization of reaction conditions, was set to 1) mix the reactants, 2) control reaction conditions and 3) sample and analyze the reaction mixtures by HPLC. The analysis was used as a guide in order to judge the success of the pentapeptide synthesis. In this manner it was possible to maximize efficiency by only working-up reactions after the yields were confirmed to be sufficient.

The purity and yields of the pentapeptides obtained are shown in Table 7. After liquid–liquid extraction, 85% of the pentapeptides were found to have purities of over 80% by HPLC. Pentapeptides that required further purification were chromatographed on disposable silica-gel tubes and the yields of over 80% were obtained. As a result, a sub-library of 56 pentapeptides was accomplished.

Conclusion

By effectively using our suite of in-house automated synthesis systems, we were able to construct a library of 72 tetrapeptides and a sub-library of 56 pentapeptides, using a convergent strategy. We showed the workstation PROW to be useful for both reaction optimization and library synthesis, and that a larger library of pentapeptides could easily be prepared.

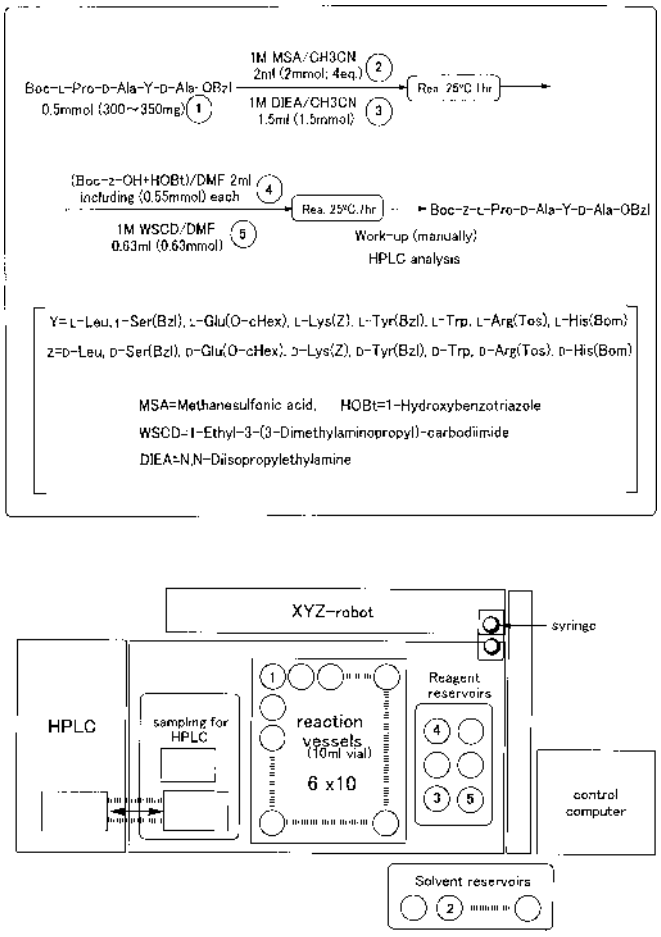

Fig. 4. Parallel Synthesis of a Pentapeptide Sub-Library Using PROW

Experimental

Computer-assisted automated synthesis workstations (TAFT, ASTRO, ASRA-COL, FUTOSHI, PROW) systems (EASY) were used to synthesize di-, tetra-, and pentapeptides in solution. NMR spectra were measured on a Varian Gemini-200 or a JOEL JNM-GX400FT NMR spectrometer (in $CDCl_3$). Solvents were of special or first grade from Wako Pure Chemical Industries Ltd., and the starting amino acid derivatives were commercially available reagents from Peptide Institute Inc.. Column chromatography was carried out on Silica gel 60 (70—230 mesh, ASTM, Merck).

Study on the Possibility of Racemization Each of the randomly selected tetrapeptides (1.5 mg) was weighed into a glass tube, then 6 N HCl was added and the mixture was heated at 110 °C for 24 h. After evaporation of the solvent, the residue was treated with distilled water (0.5 ml) to obtain a sample for analysis. Amino acid analysis for each sample was carried out using a Hitachi HPLC system (L-6200) with standard L-alanine (retention time=4.74 min) and D-alanine (retention time=5.90 min) under the following conditions; column, SUMI-CHIRAL OA-5000; column size, 4.6×150 mm; column temperature, ambient; mobile phase, 1 mM copper sulfate aqueous solution; flow rate, 1.0 ml/min; analytical wavelength, UV (254 nm); sample injection volume, 10 μl.

General Procedure for a Peptide Bond Formation. a) Synthesis of Dipeptide Benzyl Esters The procedure for peptide bond formation between Boc-L-Lys(Z) and Boc-D-Ala-OBzl is described as a typical example. Table 1 lists the chosen subroutine program, and the conditions for the reaction (reaction time, temperature, and other information) were input to the subroutine [START]. Subroutine program titles are given in square brackets below. Boc-D-Ala-OBzl (112 g; 400 mmol), and acetonitrile (300 ml) were put in the reaction flask (RF). Then from the reagent reservoir 1 (RR1), 2 M MSA in acetonitrile (500 ml; 1 mol) was added with stirring at room temperature [RF-ST-ON] [RR1-RF], before being warmed up to carry out the deprotection [RF-LF-UP, REA1 (40c, 60 m)]. After cooling the solution to 0 °C, 1 M DIEA in acetonitrile (600 ml; 600 mmol) was added from RR2, and stirred for 5 min [RF1-T-ON (0c), RR2-RF, MATU]. Meanwhile in separatory funnel (SF), Boc-L-Lys(Z) (240 g; 400 mmol) and HOBT monohydrate (59.4 g; 440 mmol) were dissolved in dimethylformamide (200 ml) and transferred to RF [SF-RF]. Then WSCD (68.3 g; 440 mmol) in acetonitrile (200 ml) was added from RR3, and reaction was carried out at 25 °C in RF for 10 h [RR3-RF, RF1-T-OF, REA1 (25c, 600 m)]. The excess solvent was

Table 7. Yield and QC data of Pentapeptides

	y****	s****	h****	w****	k****	e****	r****	l****
PaYa		**sPaYa** 51 120 28	**hPaYa** 100 480 100	**wPaYa** 61 353 80	**kPaYa** 82 327 88	**ePaYa** 81 433 47	**rPaYa** 89 440 52	**lPaYa** 82 330 81
PaSa	**yPaSa** 89 410 91		**hPaSa** 88 405 92	**wPaSa** 86 404 99	**kPaSa** 66 330 74	**ePaSa** 63 302 36	**rPaSa** 87 400 95	**lPaSa** 84 285 77
PaHa	**yPaHa** 100 450 94	**sPaHa** 100 400 91		**wPaHa** 89 400 90	**kPaHa** 86 447 92	**ePaHa** 88 440 96	**rPaHa** 82 357 87	**lPaHa** 70 357 87
PaWa	**yPaWa** 100 250 56	**sPaWa** 100 280 69	**hPaWa** 87 420 96		**kPaWa** 82 426 95	**ePaWa** 85 417 98	**rPaWa** 96 432 94	**lPaWa** 88 325 87
PaKa	**yPaKa** 96 210 44	**sPaKa** 65 260 59	**hPaKa** 68 250 52	**wPaKa** 65 325 73		**ePaKa** 52 208 45	**rPaKa** 98 420 94	**lPaKa** 81 250 58
PaEa	**yPaEa** 64 135 29	**sPaEa** 100 350 84	**hPaEa** 81 354 77	**wPaEa** 89 400 95	**kPaEa** 56 319 69		**rPaEa** 85 290 49	**lPaEa** 56 174 45
PaRa	**yPaRa** 80 370 74	**sPaRa** 92 400 86	**hPaRa** 100 440 87	**wPaRa** 94 470 89	**kPaRa** 89 463 91	**ePaRa** 86 488 90		**lPaRa** 89 320 74
PaLa	**yPaLa** 100 290 71	**sPaLa** 85 370 85	**hPaLa** 100 350 86	**wPaLa** 87 390 84	**kPaLa** 57 262 32	**ePaLa** 47 235 61	**rPaLa** 90 410 86	

..... Low purity

Small letter - D-Amino acid
Large letter - L-Amino acid

Abbreviation	
First QC (%)	
Amount (mg)	Yield (%)

removed under reduced pressure [CON1 (50c, 300 min), RF-LF-DN], and the residue was dissolved in ethyl acetate (450 ml), and 5% $NaHCO_3$ aq. (800 ml) [RS4-RF, RS2-RF]. The mixture was stirred at high speed before being transferred to SF. The standard extraction procedure (washing with 0.2 N HCl acid (500 ml), and water (500 ml)) [RF-MIX to RF-SF, ALARM] was then performed and the organic extract was transferred to the collection flask (CF) before being removed manually from the automated apparatus. Finally, the RF and lines were washed and dried [SR1-DR to END]. Excess solvent was evaporated from the extract to give white crystals, which were filtered (11G3 glass filter), washed twice with diisopropylether (500 ml) and dried to give 206 g (95%) of Boc-L-Lys(Z)–D-Ala-OBzl.

b) General Synthesis of Dipeptide Carboxylic Acid by Saponification The procedure for saponification of Boc-L-Lys(Z)–D-Ala-OBzl is described as a typical example. Table 2 lists the chosen subroutines, and the conditions for the reaction (reaction time, temperature, and other information) were input to the subroutine [START]. Boc-L-Lys(Z)–D-Ala-OBzl (108 g; 200 mmol), and methanol (600 ml) were put in the RF. Then from RR1, 1 M sodium hydroxide aqueous solution 240 ml (240 mmol; 1.2 eq) was added at 0 °C [RF-ST-ON, RF1-LF-UP, RF1-T-ON (0c), RR1-RF]. The solution was stirred at room temperature for 300 min. [RF1-T-OF, REA1 (25c, 300 m)], then cooled to 0 °C, before 1 M hydrochloric acid 240 ml (240 mmol) was added from RR3. After stirring for 5 min [RF1-T-ON (0c), RR3-RF, RF1-T-OF] the excess solvent was removed [CON1 (40c, 60 min)], and the residue was maintained at 10 °C [RF1-T-ON (10c), MATU]. From SF, 5% $NaHCO_3$ aq. (400 ml) was added to the residue under rapid stirring to separate the pure product as a salt from the by-products in the organic layer [SF-RF to SF-DR]. Then, after neutralization with 1 N hydrochloric acid (300 ml), the expected dipeptide carboxylic acid was extracted from the aqueous layer with ethyl acetate (450 ml) followed by standard work-up [SR1-RF to SF-CF, ALARM]. The CF was manually removed from the apparatus, and the solvent removed to give Boc-L-Lys(Z)–D-Ala-OH, 110 g (90%) as the dicyclohexylamine salt.

c) Catalytic Hydrogenation of Boc-L-Glu(O-cHex)–D-Ala-OBzl A solution of Boc-L-Glu(O-cHex)–D-Ala-OBzl (78.3 g, 160 mmol; 20 mmol for each cycle×8 cycles) in methanol (320 ml) was set in the RR1. A solution of ammonium formate (31.9 g, 480 mmol; 60 mmol for each cycle×8 cycles) in methanol (320 ml) was set in the RR2 and 10% Pd–C (1.5 g) was suspended in methanol (20 ml) in the RF. For the first cycle, the dipeptide solution (40 ml, 20 mmol) was transferred to the RF from RR1, followed by the ammonium formate solution (40 ml, 60 mmol) from RR2. The reaction mixture was well mixed by shaking at room temperature for 2 h, and then the solution containing the deprotected benzyl ester was transferred from the RF through a teflon filter to the CF. The RF was washed twice with methanol (40 ml) and the washings were combined in the CF. The methanol solution in CF was evaporated and then the catalyst in the RF was washed with 1 M formic acid solution in methanol (80 ml), followed by methanol (80 ml). For the next cycle, the reagents were transferred to the RF, and the same sequence of subroutines was run for an additional 7 cycles. During the runs, 1.5 g of 10% Pd–C was renewed after every 3 cycles. The extracted organic solvent was dried and removed to give 46.3 g (71%) of Boc-L-Glu(O-cHex)–D-Ala-OH as a oil.

d) General Procedure of the Peptide Bond Formation The procedure for the peptide bond formation between Boc-L-Lys(Z)–D-Ala-OH and Boc-L-Tyr(Bzl)–D-Ala-OBzl is described as a typical example. Table 4 lists the chosen subroutine program, and the conditions for the reaction (reaction time, temperature, and other information) were input to the subroutine [START]. Boc-L-Tyr(Bzl)–D-Ala-OBzl (6.39 g; 12.0 mmol) was put as a powder in the RF1, and Boc-L-Lys(Z)–D-Ala-OH (5.42 g; 12.0 mmol) and HOBT (2.02 g; 13.2 mmol) were dissolved in DMF (10 ml) in the RF2 [RF1-ST-ON, RF2-ST-ON]. Then, from RR1, 2 M MSA in acetonitrile solution, 15 ml (30 mmol; 2.5 eq), was added at room temperature [RR1-RF1]. The solution was then stirred at 40 °C for 60 min [RF1-LF-UP, REA1 (40c, 60 m)]. After cooling the solution to 0 °C, 0.9 M DIEA in acetonitrile, 20 ml (18 mmol), was added from RR2, and stirred for 5 min [RF1-T-ON (0c), RR2-RF1, MATU]. Then at 0 °C, the solution in RF2 was added to RF1 while stirring was continued [RF2-RF1, RF6-RF2, RF2-MIX, RF2-RF1, RF2-ST-OF]. After adding WSCD (2.05 g; 13.2 mmol) in acetonitrile (5 ml) from RR3, the condensation reaction was carried out at 25 °C in RF1 for 10 h [RR3-RF1, RF1-T-OF, REA1 (25c, 600 m)]. The excess solvent was removed under reduced pressure [CON1 (40c, 60 min), RF1-LF-DN], and the residue was first dissolved in THF (20 ml), followed by ethyl acetate (30 ml), and finally 5% $NaHCO_3$ aq. (40 ml) [RS5-RF1, RS4-RF1, RS2-RF1]. The mixture was rapidly stirred and transferred to SF where the standard extraction procedure was performed (washing with ethyl acetate, 40 ml, followed by acid (0.2 N HCl, 40 ml), and water (40 ml)) [RF1-MIX, BKTETRANK, RF1-ST-OF, ALARM]. The collected product was then removed manually from RF3, and the RFs and lines were washed and dried [SR1-DR to END]. The excess solvent was evaporated and the crystals of Boc-L-Lys(Z)–D-Ala–L-Tyr(Bzl)–D-Ala-OBzl were collected on an 11G3 glass filter, washed twice with diisopropylether and dried to give 9.53 g (91.7%). ^{1}H-NMR (200 MHz, $CDCl_3$): 1.11—1.14 (d, 3H), 1.17—1.20 (d, 3H), 1.23—1.61 (m, 15H), 2.84—3.16 (d-d, 2H), 3.13—3.15 (d, 2H), 4.00—4.08 (m, 1H), 4.21—4.41 (t, 1H), 4.49—4.68 (m, 2H), 5.01 (s, 2H), 5.07 (s, 2H), 5.13 (d, 2H), 5.35 (d, 1H), 6.84—7.40 (m, 19H).

e) General Procedure for the Peptide Bond Formation to Form Pentapeptides The parallel synthesis of a sub-library of 56 pentapeptides, described by the formula Boc-Z–L-Pro–D-Ala–Y–D-Ala-OBzl, where Z is randomly selected from D-Leu, D-Ser(Bzl), D-Glu(O-cHex), D-Lys(Z), D-Tyr(Bzl), D-Trp, D-Arg(Tos), D-His(Bom), and Y is randomly selected from L-Leu, L-Ser(Bzl), L-Glu(O-cHex), L-Lys(Z), L-Trp, L-Arg(Tos), and L-His(Bom), is shown in Fig. 4, as a typical example. First, one of the 7 starting tetrapeptides (Boc-L-Pro–D-Ala–Y–D-Ala-OBzl was weighed into each of the reaction vessels (0.5 mmol; 300—350 mg), and the stock solution of 1 M MSA in acetonitrile (2 ml for each reaction (2 mmol; 4 eq) was set in a dispense reservoir. A stock solution of DIEA in acetonitrile (1.5 ml per reaction (1.5 mmol)), WSCD in DMF (0.63 ml per reaction (0.63 mmol), and Boc-Z (0.55 mmol per reaction) plus HOBt (84 mg per reaction (0.55 mmol)) in DMF (2 ml per reaction) were set in the reagent reservoirs. Following the procedure in Fig. 4, the robot delivered reagents to the reaction vessels automatically, and after the reaction, each sample was analyzed by HPLC. The reaction mixture was extracted manually from ethyl acetate (10 ml) and 5% $NaHCO_3$ aq. (15 ml), followed by additional extraction (ethyl acetate (10 ml)). The combined organic extract was analyzed by HPLC, and if the purity of the product was over 80%, it was simply evaporated, whereas if the purity was under 80%, it was chromatographed on disposable silica-gel tubes. After evaporation and crystallization, the expected pentapeptides were obtained in about 20—80% yield (100—500 mg).

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