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Library generation through successive substitution of trichlorotriazine

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Summary

The decreasing reactivity of tri-, di- and monochlorotriazine was utilized for the solid-phase construction of a combinatorial library with three randomized positions, using 20 amino acids and 50 amines as building blocks. The first chlorine atom was selectively substituted by coupling a large excess of trichlorotriazine to the support-bound amino acid, thus avoiding simultaneous substitution of the second chlorine. The second and third diversity positions were selectively introduced by coupling amines at different temperatures. Mixtures of model compounds were synthesized and analyzed, showing the correct representation of all expected components. A library composed of 12 000 compounds was generated using this method.

Introduction

The generation of molecular diversity based on nonpeptidic structures is being increasingly utilized for both drug discovery and basic research (for recent reviews in this field see Refs. 1–5). Nonpeptidic structures can be tailored specifically for particular biological interactions, and, moreover, they are suitable candidates for metabolically stable and bioavailable pharmaceuticals. The predominant method used for the generation of molecular diversity is solid-phase synthesis. Contrary to the solid-phase synthesis of peptides, which has been used and optimized for decades, the synthesis of small organic molecules on the solid phase is still in its infancy.

Currently, there are three principal approaches to the combinatorial synthesis of organic molecules on a solid phase: (i) the stepwise combination of building blocks in several separate reactions; (ii) the attachment of building blocks to a preformed molecular scaffold, the reactive groups (attachment points) of which have to be orthogonally protected in order to enable the selective (i.e., site-specific) introduction of building blocks [6–8]; and (iii) the use of reactions that selectively expose one reactive group while simultaneously introducing another diversity position, e.g. the reaction of intramolecular anhydrides with amines, alcohols, etc. [8–10].

The three symmetrically positioned electrophilic centers of trichlorotriazine enable an alternative method for the selective introduction of diversity positions through the successive substitution of the chlorine atoms. This feature of trichlorotriazine is well known and widely utilized, e.g. to dye fabric in the textile industry. Triazines have also been tested as functionalizing agents for solid supports [11].

One-bead–one-compound libraries [12–14] are screened either in solid-phase binding assays, or in solution. In the first case, the library beads are incubated with the target molecule, and binding is detected by a colorimetric reaction, fluorescence, or autoradiography. Positively reacting beads are selected, and the compounds are released from the beads, followed by structure determination. For the solution assay, the compound has to be released from the bead, and the structure of the positively reacting compound is then determined either from the compound in solution, from the second or third [15] copy of the compound, or its code [16–21] still attached to the bead, or from the compound remaining on the bead after the release.

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An alternative library strategy involves compound mixtures with randomized and defined positions in the library molecule [22,23]. The individual compounds responsible for an observed biological activity in a particular mixture are identified based on the building blocks at the defined positions, either through an iterative process, or a positional scanning algorithm.

Materials and Methods

General

Solid-phase syntheses were performed on polyethylene grafted copoly(styrene–1% divinylbenzene) 220 μm resin (PEG/PS, Perseptive, Bedford, MA, U.S.A.). Fluorenylmethyloxycarbonyl (Fmoc) amino acids with standard side-chain protecting groups were obtained from Advanced ChemTech (Louisville, KY, U.S.A.) or Propeptide (Vert-le-Petit, France). Amines, anilines and hydrazines used in randomizations, diisopropyl carbodiimide (DIC), diisopropylethylamine (DIEA), *N*-hydroxybenzotriazole (HOBt), phenol, piperidine, anisole and trifluoroacetic acid (TFA) were obtained from Aldrich Chemical (Milwaukee, WI, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Anhydrous dioxane was obtained from Aldrich Chemical. High-purity solvents (Baxter, McGaw Park, IL, U.S.A.) were used without further purification.

Analytical HPLC was carried out on a Hitachi LC system (L-6200 Intelligent Pump, AS-2000 autosampler) with a L-3000 diode array detector (Hitachi Ltd., Tokyo, Japan) using Vydac Peptide and Protein C_{18} analytical column (4.6×250 mm, 5 μm, 1 ml/min) (The Separation Group, Hesperia, CA). The analytical gradient was run from water containing 0.07% TFA to 60% of acetonitrile (MeCN)/water (0.07%TFA) in 30 min. UV/VIS Absorption spectra were recorded on a Hewlett-Packard HP 8452A Diode-Array spectrophotometer (Palo Alto, CA, U.S.A.) using a 1-cm quartz cuvette. Ion-spray mass spectra were measured on a triple quadrupole PE-Sciex API III+ mass spectrometer (Perkin-Elmer/Sciex, Thornhill, ON, Canada) with an articulated ion-spray sample-inlet system.

Linker attachment

The solid support (18 g PEG-PS·HCl, substitution 0.58 mmol/g, size 220 μm, Perseptive, #CRD 4240) was swollen in DMF (120 ml) for 20 min, followed by treatment with 10 % DIEA in DCM (2×120 ml, 2 min). The solid support was washed with DCM (2×100ml), DMF (1×100ml) and 5% HOBt in DMF (1×100 ml). To a solution of Fmoc-L-methionine (11 g, 30 mmol) and HOBt (4 g, 30 mmol) in DMF (100 ml), DIC (4.7 ml, 30 mmol) was added and the mixture stirred for 20 min at room temperature. The activated methionine was added to the resin, and the suspension was agitated with nitrogen for 1 h at room temperature or until the ninhydrin test was negative, indicating complete coupling. The solid support was washed with DMF (2×100 ml), DCM (1×100 ml) and DMF (1×100 ml). The Fmoc group was removed by treatment with 50% piperidine in DMF (2×50 ml) for 30 min, and the resin washed with DMF (4×100 ml). The substitution with methionine, determined by UV measurement of the dibenzofulvene-piperidine adduct (λ_{max} 302 nm) formed during the deprotection, was found to be 0.49 mmol/g.

First randomization

The resin was split into 20 equal portions and distributed in Wheaton glass vials (0.9 g, 0.44 mmol per vial). The Fmoc-protected amino acids used for the first randomization are listed in Table 1. Amino acid side chains were protected with the *tert*-butyloxycarbonyl (Boc) group (Lys, Orn), the *tert*-butyl (*t*-Bu) group (Ser, Tyr, Thr, Asp, Glu), the triphenylmethyl (Trt) group (Asn, Gln, His) or the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group (Arg). To a solution of protected amino acid (1.5 mmol) and HOBt (203 mg, 1.5 mmol) in DMF (5 ml), DIC (237 μl, 1.5 mmol) was added, and the reaction mixture was shaken for 20 min at room temperature. The activated amino acid solutions were added to the resins and shaken at room temperature for 3 h, or until the ninhydrin test was negative. The 20 resin portions were then combined and washed with DMF (2×100 ml), DCM (1×100 ml), and DMF (1×100 ml). The Fmoc group was removed by treatment with 50% piperidine in DMF (2×50 ml, 1+15 min), and the resin washed with DMF (4×100

TABLE 1
PROTECTED AMINO ACIDS USED IN THE FIRST RANDOMIZATION

Number	Building block[a]	M.W.	Amount[b]
1	Gly	297.3	446
2	Ala	311.5	467
3	Leu	353.4	530
4	Ile	353.4	530
5	Phe	387.4	581
6	Ser	383.4	575
7	Thr	397.5	596
8	Tyr	459.5	689
9	Trp	426.5	640
10	Val	339.4	509
11	Arg	662.8	994
12	Asp	411.5	617
13	Asn	596.7	894
14	Glu	425.5	638
15	Gln	611.8	918
16	His	619.7	929
17	Lys	468.6	703
18	Pro	337.4	506
19	Chg	381.5	571
20	Orn	454.5	682

[a] Protected amino acids.
[b] Amount (mg) of protected amino acid used for randomization.

TABLE 2
AMINES USED IN THE SECOND RANDOMIZATION

Number	Building block	M.W.	Amount[a]
1	isopropylamine	59	50
2	butylamine	73	62
3	isobutylamine	73	62
4	cyclohexylamine	99	84
5	beta-Ala-OBut·HCl	181	154[b]
6	1-(2-aminoethyl)pyrrolidine	114	97
7	benzylamine	107	91
8	1-naphthalenemethylamine	157	133
9	tyramine	137	116
10	4-methoxybenzylamine	137	116
11	3,5-dimethoxybenzylamine	167	142
12	2-aminoindane·HCl	169	144[b]
13	1,2,3,4-tetrahydro-1-naphthylamine	147	123
14	cyclopentylamine	85	72
15	*N*-Boc-1,3-diaminopropane	174	148
16	*N*-Boc-1,4-diaminobutane	188	160
17	1-(2-aminoethyl)pyrrolidine	114	97
18	*N*-methylcyclohexylamine	113	96
19	*N*-benzylmethylamine	121	103
20	dibenzylamine	197	167
21	4-ethyl-(aminomethyl)pyridine	136	116
22	1-methylaminomethyl-naphthalene·HCl	207	176[b]
23	cyclohexylmethylamine	113	96
24	4-aminomorpholine	102	87
25	3,3-dimethylbutylamine	101	86
26	2-methoxyethylamine	75	64
27	aminodiphenylmethane	183	155
28	4-(2-aminoethyl)morpholine	130	110
29	4-methylpiperazine	100	85
30	4-(aminomethyl)pyridine	108	92

[a] Amount (mg) of amine used for randomization.
[b] DIEA (148 µl, 0.85 mmol) was added.

ml). The substitution with amino acid, determined by UV measurement of dibenzofulvene-piperidine adduct (λ_{max} 302 nm) formed during the deprotection, was found to be 0.41 mmol/g.

Scaffold (trichlorotriazine) attachment

The resin was washed with DCM (2 × 100 ml) and cooled to 2 °C for 30 min. A solution of cyanuric chloride (5.8 g, 31.3 mmol) in DCM (100 ml) was added to the resin, followed by addition of DIEA (5.5 ml, 31.3 mmol) in DCM (20 ml) over a period of approximately 15 min. The suspension was agitated with nitrogen at room temperature until the ninhydrin test was negative (ca. 1 h). The resin was washed with DCM (3 × 100 ml).

Second randomization

The resin was divided into 30 equal portions and distributed in Wheaton glass vials (0.6 g, 0.25 mmol per vial). A solution of the amine (0.85 mmol, Table 2) in DCM (5 ml) was added to the resin and the suspension was shaken for 2 h at room temperature. Vials #1–15 and #16–30 were mixed together to give group A and group B, respectively. Resin (group A and B separately) was washed with DCM (3 × 50 ml) and 1,4-dioxane (1 × 50 ml).

Third randomization

The resin (group A) was divided into 20 equal portions and distributed in V-shape 4 ml-Wheaton glass vials (0.45 g, 0.18 mmol per vial). A solution of an amine or hydrazine (5.2 mmol, Table 3) in dry 1,4-dioxane (0.6 ml) was added to each resin portion, and shaken for 2 h at 90 °C. The resins were then transferred to syringes equipped with polypropylene frit at the bottom [24]. Each syringe was washed with DCM (5 × 4 ml). The same procedure was performed with group B.

Side-chain deprotection

Each of the 40 resin portions was treated with 4 ml TFA containing 5% thioanisole, 2.5% 1,2-ethanedithiol and 5% water for 2.5 h at room temperature, washed with TFA (2 × 4 ml), DCM (5 × 4 ml), DMF (1 × 4 ml), MeOH (3 × 4 ml), and dried under vacuum for 12 h. The dry resins were stored at 5 °C.

Release of compound from resin

Resin containing the methionine linker (0.9 g resin) was treated with 4 ml cyanogen bromide solution (20 mg/ml in 0.1 N HCl) at 25 °C for 12 h in the darkness. Reactions was stopped by freezing and lyophilization. The product was extracted by methanol.

TABLE 3
AMINES AND HYDRAZINES USED IN THE THIRD RANDOMIZATION

Number	Building block	M.W.	Amount[a]
1	*tert*-butylhydrazine·HCl	124	322[b]
2	4-chlorophenylhydrazine·HCl	179	465[b]
3	4-hydrazinobenzoic acid	152	395
4	indoline	119	309
5	pyrrolidine	71	185
6	cyclopropylamine	57	148
7	adamantanemethylamine	165	429
8	*p*-toluidine·HCl	143	372[b]
9	2-(methylmercapto)aniline	139	361
10	1,2,3,4-tetrahydroisoquinoline	133	346
11	1,2,3,4-tetrahydroquinoline	133	346
12	H-Ser(*t*Bu)-O*t*Bu	217	564
13	H-Lys(Boc)-NH2·HCl	282	733[b]
14	1-aminopiperidine	100	260
15	morpholine	87	226
16	*p*-anisidine	123	320
17	3-methoxypropylamine	89	231
18	aniline	93	242
19	piperidine	85	221
20	1-methyl-4-(methylamino)piperidine	128	333

[a] Amount (mg) of amine or hydrazine used for randomization.
[b] DIEA (452 µl, 2.6 mmol) was added.

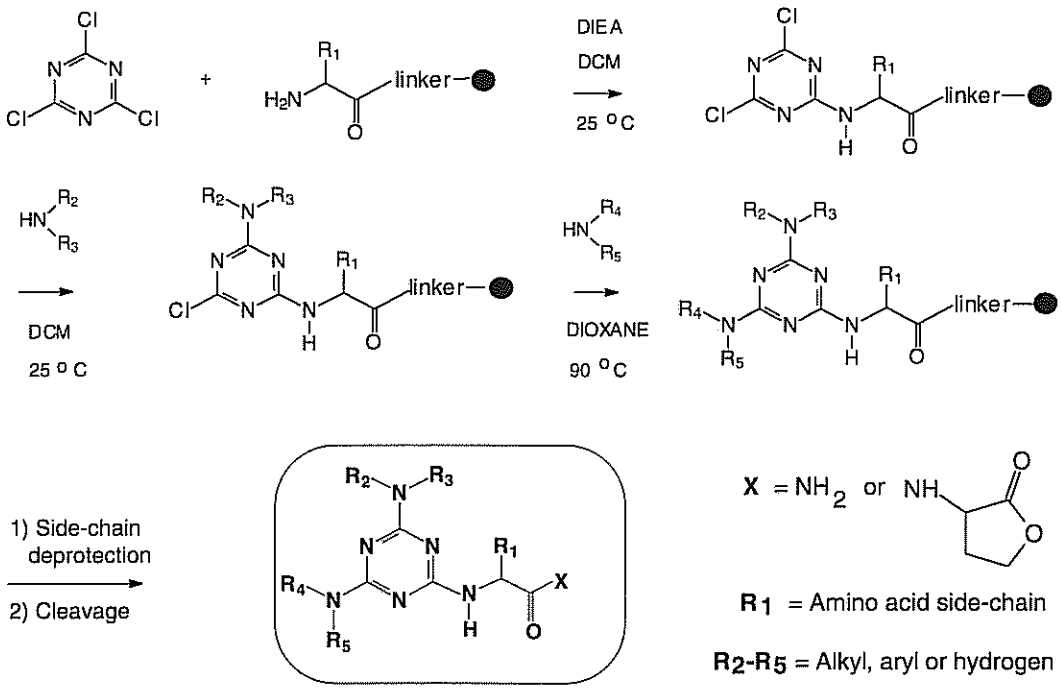

Fig. 1. Scheme of the synthesis of the triazine-based library.

Results and Discussion

Since the library was intended to be used in both solid-phase and solution assays, a selectively cleavable linker had to be found that would be stable to the TFA treatment used for the final side-chain deprotection of amino acids. Peptide bonds involving the carboxy function of methionine can be cleaved very specifically by bromocyanate. Consequently, methionine was used as a selectively cleavable linker between the library and the resin. This type of linkage to the support was used earlier for release of the compounds for mass spectrometric analysis [25].

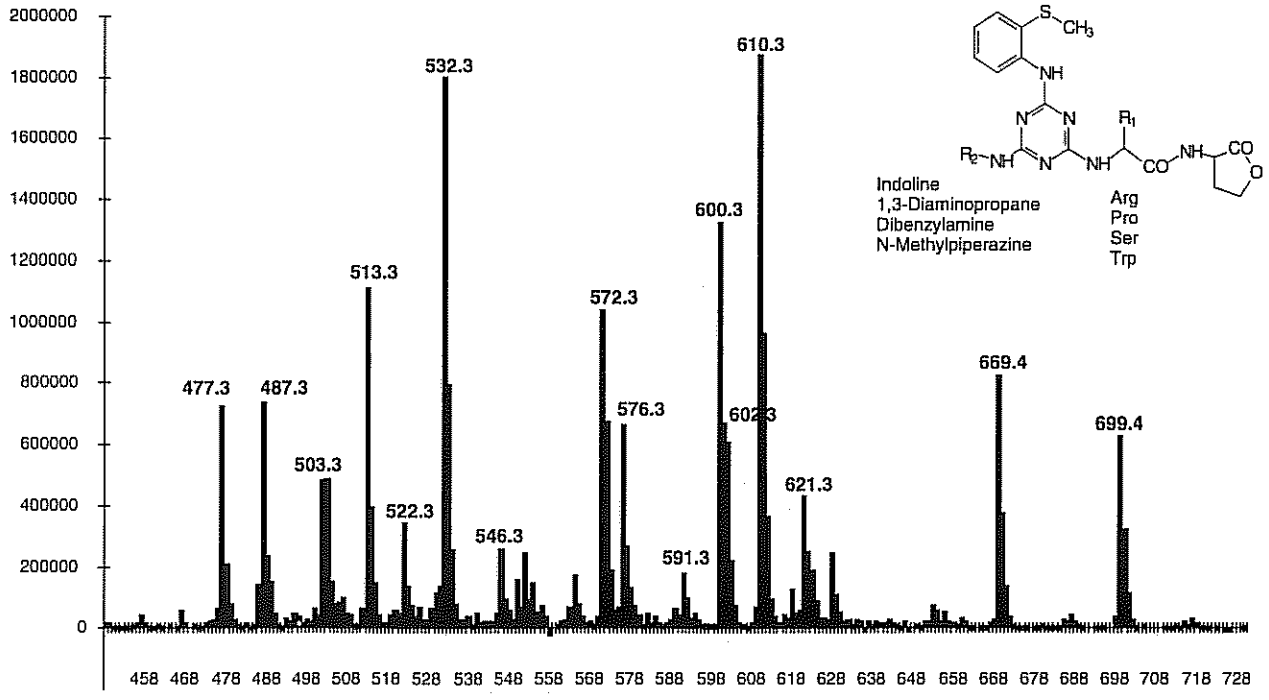

Fig. 2. Mass spectrum of a model mixture of 16 compounds synthesized using four amino acids for the first and four amines for the second randomizations, as well as 2-(methylmercapto)aniline for the substitution of the third chlorine atom of trichlorotriazine.

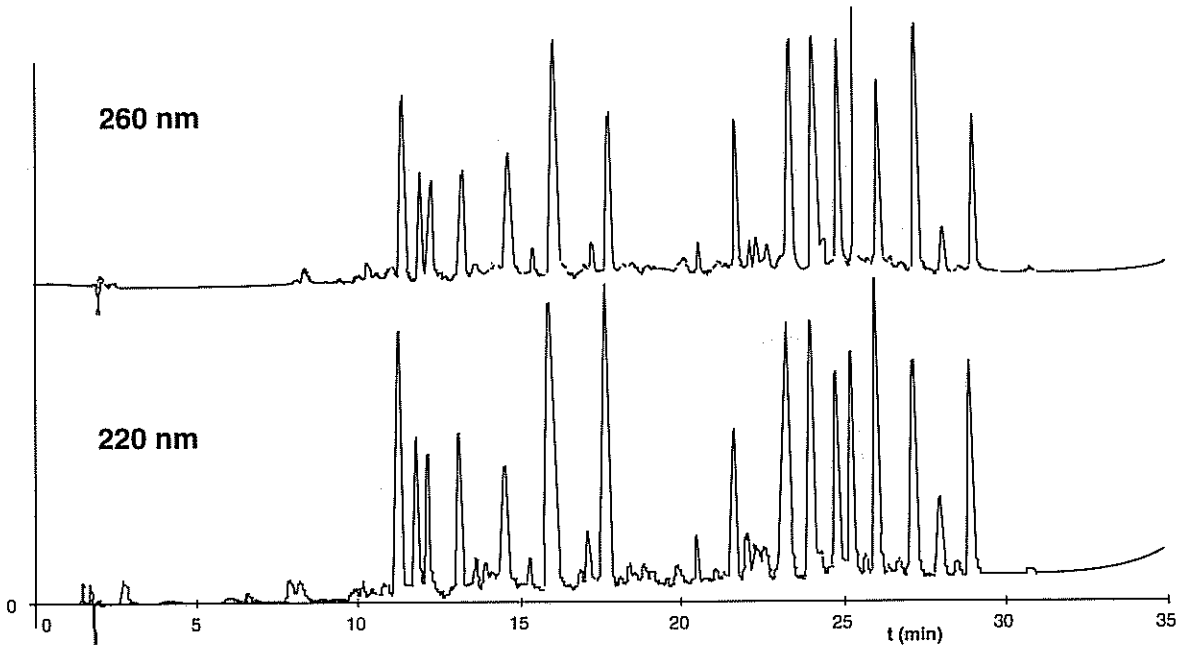

Fig. 3. HPLC traces at 260 nm and 220 nm of a model mixture of 16 compounds (see Fig. 2).

The library was synthesized using the 'split-and-mix' method described earlier [26]. The synthesis scheme is shown in Fig. 1. Although both the first and second chlorine atoms can be substituted at room temperature, the kinetics of both reactions are sufficiently different, so that the use of a large excess of trichlorotriazine in combination with the amine attached to the solid support enables the selective substitution of only the first chlorine atom under conditions normally leading to the substitution of both chlorine atoms. We have used natural and unnatural amino acids attached to the polymeric carrier, and we have never observed cross-linking (triazines bis-substituted with amino acid). The second step of library construction was the substitution of the second chlorine with primary or secondary amines. This step was also performed at room temperature, but, unlike in the previous step, an excess of amine was used in order to drive the reaction to completion. For this step it was important to select the amines which yielded clean, monosubstituted products without dialkylated side products. The third chlorine atom was substituted under elevated temperature. Since the molecule is symmetrical, the selection of amines for the second or the third randomization has no influence on the overall complexity of the created library. Therefore we could use very reactive amines (piperidine, indoline) in the third randomization, where the danger of overreaction is eliminated. Amino acids and amines selected for the first, second and third randomization are listed in Tables 1–3.

After demonstrating the feasibility of the proposed synthesis strategy through the synthesis of individual model compounds, a model mixture of 16 compounds was synthesized using four amino acids (Arg, Pro, Ser, Trp) in the first, four amines (indoline, 1,3-diaminopropane, dibenzylamine, *N*-methylpiperazine) in the second, and one amine (2-methylmercaptoaniline) in the last step. The mixture was released from the resin by cyanogen bromide and analyzed by mass spectrometry (Fig. 2) and HPLC (Fig. 3). The HPLC chromatogram and mass spectrum clearly demonstrate the presence and reasonable distribution of the 16 mixture components.

A library of medium complexity (12 000 compounds) was synthesized using the building blocks listed in Tables 1–3. The number of compounds in a mixture can be adjusted by subpooling in the last steps of library synthesis. The synthesis described in the experimental part used two subpools after the second randomization and resulted in 40 pools of 300 compounds. The screening results obtained with this library will be reported elsewhere.

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