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Antibacterial single-bead screening

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Abstract—Triazine based antibiotics were prepared by the attachment of cyanuric chloride onto a Marshall-type safety catch linker, followed by successive aromatic nucleophilic substitutions, linker activation and nucleophilic cleavage. High-loading dendrimer beads allowed the release of sufficient amount of compound from a single bead to give clear inhibition.

Bead-based screening, exploiting the one-bead one-compound concept of split and mix combinatorial library synthesis, is a powerful method for the synthesis and screening of large libraries either on or off beads.[1] Screening peptide-based libraries has been carried out with the peptide tethered to the solid support, whereas libraries of small organic compounds have usually been screened in solution after cleavage, where the molecule is fully exposed in the assay medium.[2] Among the numerous solution-phase assays available, gel-permeation assays for antimicrobial screening can be easily adapted to the screening of bead-based combinatorial libraries.[3] Jayawickreme et al. presented the first evidence that single-bead activity from a large peptide-based library could be detected following cleavage from single beads and gel based assay[4] and this was followed by the screening of a large non-peptidic type library in a similar lawn assay.[5] These assays using porous gel matrices (primarily agar or agarose) consisted of spreading the library beads across the gel matrix and cleaving the compounds from the solid-phase support into the gel containing the appropriate cells. Zones of inhibition corresponding to the lack of cell growth indicate the presence of active compounds.[6] However, a critical issue is the limited amount of compound available on single beads. For instance, a standard single polystyrene bead bearing approximately 500 pmol of compound will only yield a 5 μM solution in a 100 μl volume. This limitation may be critical to the viability of the assay and its sensitivity. Here, we report on the use of high-loading dendrimerised resin beads for single-bead antibacterial screening.

Keywords: beads; dendrimers; triazines; antibiotic; linker.
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1. Results and discussion

Our studies targeted compounds containing a decorated 1,3,5-triazine. Such structures have recently been found to inhibit DNA Gyrase,[7] an enzyme responsible for DNA super-coiling. This represents an attractive antibacterial target because it is vital to bacteria yet has no direct counterpart in mammals. Moreover, 1,3,5-triazine represents a useful template for the construction of heterocycles and has been widely reported for library generation.[5,8] We recently reported a novel 1→3 *C*-branched isocyanate-type monomer which was used to prepare high-loading beads via a divergent dendrimerisation process.[9] Using 250–300 μm 1% DVB polystyrene resin beads generation 1.0 and 2.0 resin-bound dendrimers were prepared with a loading of 46 and 119 nmol/bead, respectively (Fig. 1).

The safety-catch linker of Marshall and Liener[10] was chosen for our synthesis as it is easily converted into the activated nucleophile-sensitive arylsulfone using mild oxidative conditions. The utility of this strategy has been reported for the synthesis of peptides,[10,11] ureas,[12] and heterocyclic compounds.[13] Prior to embarking on the solid-phase library synthesis, solution-phase models were investigated. Benzylamine, used as a starting point for the synthesis, was reacted with the Marshall-type linker **1** using DCC and HOBt (Scheme 1). Reaction of **2a** with cyanuric chloride in THF afforded the 3,5-dichloro-triazine **3a** in 98% yield (no product was formed when the reaction was performed in DMSO or DMF, which are usually the first choice solvents for dendrimerised resins due to their efficient swelling of the resins which thus maximises site accessibility and minimises cross-coupling). The first aromatic nucleophilic substitution with *p*-fluoroaniline worked successfully in both THF and DMF although further substitution of the remaining chloride appeared to be a problem. A 0.2 M solution of *p*-fluoroaniline in THF reacted with

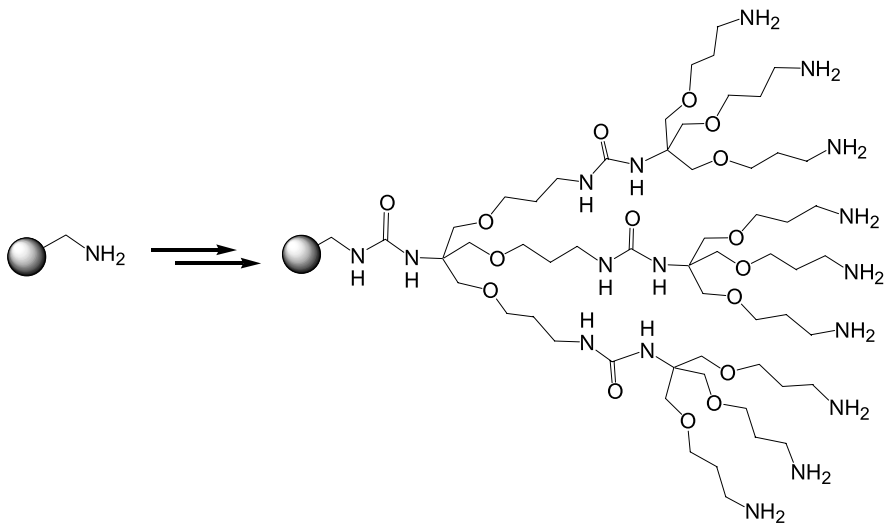
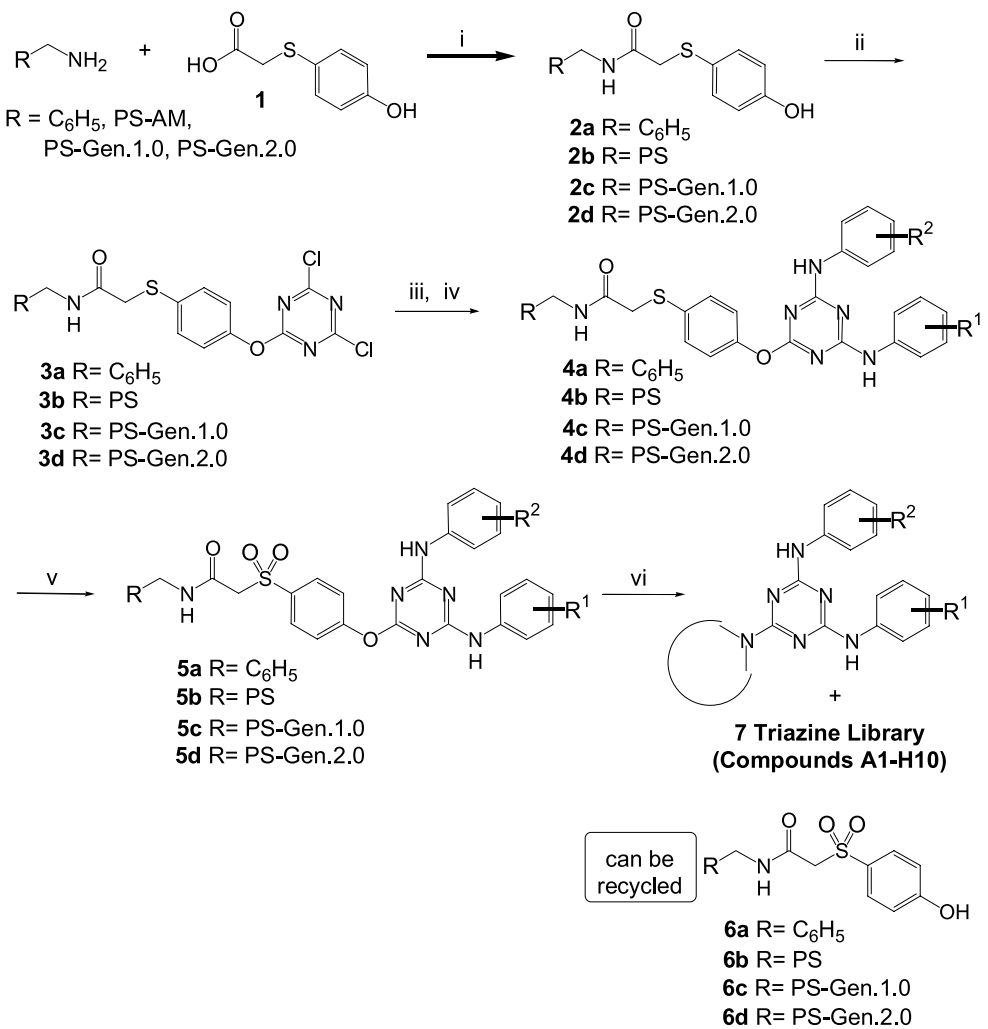


Figure 1. Generation 2.0 resin-bound dendrimer.



Scheme 1. Solution model and solid-phase triazine synthesis strategy (PS=Polystyrene resin). (i) HOBt, DIC/DMF; (ii) cyanuric chloride/THF; (iii) aniline derivative (R^1), DIPEA/DMF or DMF/THF (1:1); (iv) aniline derivative (R^2), DIPEA/DMF; (v) CH_3CO_3H/DCM; (vi) morpholine or piperidine or *N*-methylpiperazine or pyrrolidine/DMSO, 100°C. See Figure 3 for the monomers used in library synthesis.

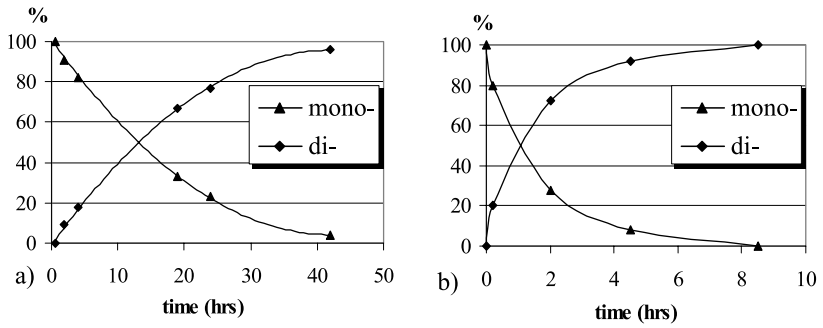


Figure 2. (a) Rate of reaction of *p*-fluoroaniline onto **3a**. Mono-substitution (complete after 30 min) versus di-substitution. (b) Rate of addition of *p*-methoxyaniline onto 3-chloro-5-(*p*-fluororaniline)-triazine.

Table 1. Rate of triazine cleavages at 60, 100, and 130°C

T (°C)	Arylsulfide **4a**[a]	Arylsulfone **5a**[a]
60	Not cleaved	10 h
100	~120 h	1
130	~40 h	<40 min

[a] Time required for complete cleavage as determined by LC/MS.

Table 2. Secondary amine mediated triazine cleavage at 100°C

Amine	Time[a] (min)
Piperidine	40
N-Methylpiperazine	45
Pyrrolidine	5

[a] Time required for complete cleavage as determined by LC/MS.

3,5-dichlororo-triazine **3a** (3 equiv. of *p*-fluoroaniline with respect to **3a**; i.e. library conditions) and the relative rates of mono and di-analine substitution were monitored by LC/MS (Fig. 2(a)). The optimal reaction time was observed to be 30 min, at which time 100% of the mono-substituted triazine was formed. Longer exposure resulted in additional substitution.

Similarly, the addition of a 0.2 M solution of *p*-methoxyaniline in DMF onto the 3-chloro-5-(*p*-fluororaniline)-triazine scaffold was monitored (Fig. 2(b)). The reaction reached completion after 10 h. As expected *p*-methoxyaniline reacted much faster than *p*-fluororaniline due to the enhanced electron-rich character of the aniline. On going from the first to the second aromatic substitution, the electrophilic character of the aromatic triazine ring is reduced thus for library synthesis purposes, it was reasoned that the first set of anilines to be reacted onto 3,5-dichloro-triazine scaffold should be the least electron-donating, while the second set much more electron rich.

Oxidation of the arylsulfide **4a** was performed with peracetic acid in DCM. The electron-donating character of the sulfide increases the electron density within the aromatic triazine system, which makes it more inert towards nucleophilic attack. Once oxidised, the electron-withdrawing character of the sulfoxide renders the triazine ring much more electrophilic and thus more labile to aromatic nucleophilic substitution at the *ipso* position. Initial oxidation of the sulfide to the sulfonyl was found to be fast (<10 min) followed by further oxidation to the sulfone. After 3–4 h, the ratio of sulfone/sulfonyl as determined by LC/MS was 90:10. This did not increase despite adding more oxidising agent. However, the presence of the sulfoxide product was not a problem as it is also electron withdrawing and was displaced efficiently.

The non-oxidised and oxidised forms of 3-(*p*-fluoroaniline)-5-(*p*-methoxy aniline)-triazine derivatives (**4a** and **5a**, respectively) were reacted with morpholine in DMSO at 60, 100, and 130°C and monitored by LC/MS (Table 1) and showed a significant increase in reactivity when the linker was activated.

Cleavage with a range of cyclic secondary amines proceeded with high efficiency at 100°C (Table 2), all triazines being completely cleaved within 1 h.

Following optimisation in solution, the chemistry was transferred to polystyrene resin and gave purities of cleaved

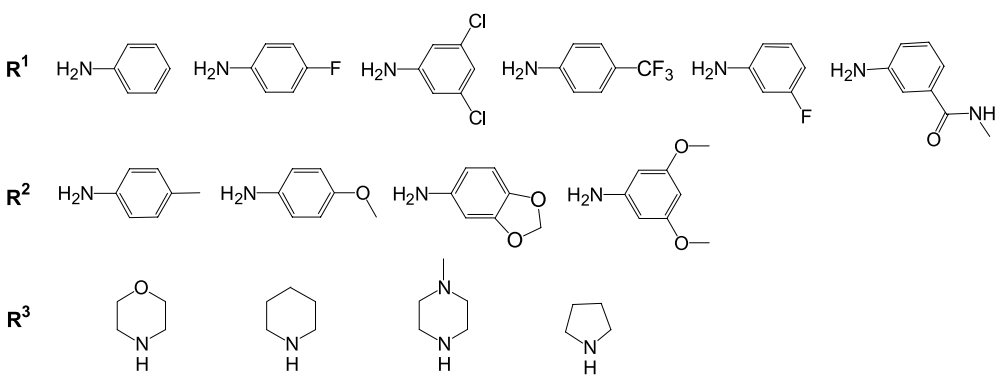


Figure 3. Building blocks used for triazine library synthesis.

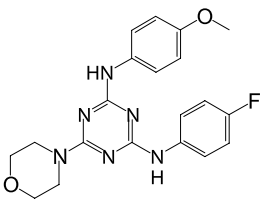


Figure 4. Structure of the triazine chosen for single-bead loading quantification.

Table 3. Single-bead loading determinations (results shown in nmol/bead)

	Gen. 0.0	Gen. 0.0 recycled	Gen. 1.0	Gen. 2.0
Bead 1	15.9	10.4	24.0	48.6
Bead 2	16.9	14.2	23.3	47.4
Bead 3	16.3	11.5	21.2	51.3
Bead 4	17.2	11.8		
Average	16.6 (92%)	12.0 (67%)	22.8 (50%)	49.1 (41%)

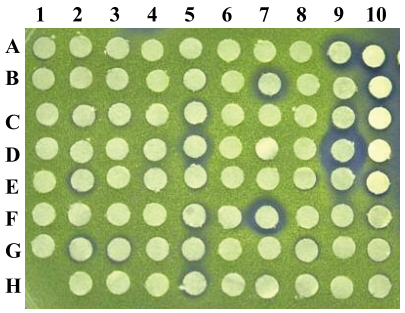


Figure 5. Zone-based diffusion assay.

compounds between 83 and 98%. Triazine cleavage resulted in the formation of the phenolic resin **6b**, which could be reused by repeating all synthetic steps (i.e. addition of cyanuric chloride followed by subsequent aniline additions) to afford cleaved triazines with purities between 58 and 63%, but the pre-oxidised linker made all displacements much faster and a little less discriminating.

Initial synthesis and cleavage from generation 1.0 dendrimerised resin afforded the expected triazines with purities between 55 and 75%. Close monitoring of the reaction cleavage with all four cyclic amines (R^3, Fig. 3) showed that in most cases a compound of mass M+16 was being generated at a much faster rate than the expected triazine (which eluted before the desired triazine on LC/MS). This suggested that the impurity was an oxidised form of the triazine due to the long exposure to peracetic acid. The *N*-oxide triazine would also withdraw electron density from the triazine system, which would result in faster nucleophilic attack and cleavage compared to the non-oxidised triazine. To verify this, the oxidation step was carried out for 7 rather than 18 h in order to reduce N-oxidation. Cleavage in this case gave all triazines in purities between 81 and 94%. The beads were found to be robust throughout the synthesis, even to the high temperature cleavages.

Construction of the single-bead based triazines on generation 2.0 resin began by reacting a 0.5 M solution of cyanuric chloride (50 equiv.) with resin-bound linker **2d** followed by amine displacement as shown in Scheme 1. Oxidation to the sulfone with peracetic acid was followed by nucleophilic aromatic substitution with morpholine in DMSO at 100°C to afford the expected triazine **7** in 77% purity (HPLC, λ=254 nm) (Fig. 4).

Based on an HPLC calibration analysis, loading measurements were carried out on the triazines **5b**,**c**,**d** (Table 3). Cleavage from a generation 2.0 single bead released 49.1 nmol of product, corresponding to a 41% yield based on the full loading of generation 2.0 resin-bound dendrimer.

A 96-compound library ($6R^1\times4R^2\times4R^3$, Fig. 4) was synthesised starting from the 3,5-dichloro-triazine resin **3**. The resin was distributed into a 96-well filter-plate where reaction steps were carried out. Once the linker was activated beads were transferred into a Bohdan Miniblock™ to allow cleavage at 100°C. Removal of the solvent and excess of amines under vacuum using a Genevac™ afforded the triazine derivatives. The library was then split into two daughter plates for quality assessment and

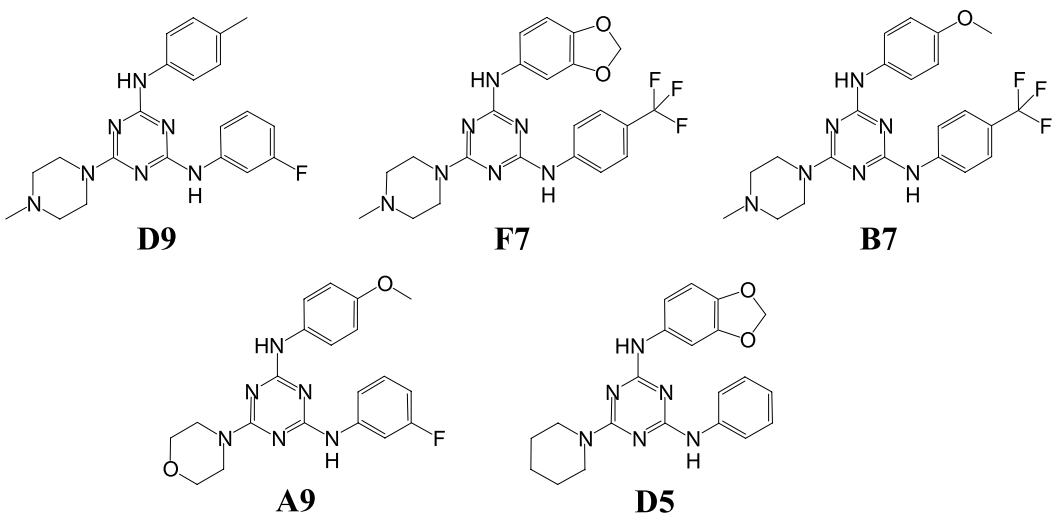


Figure 6. Most active library members showing antibacterial activity.

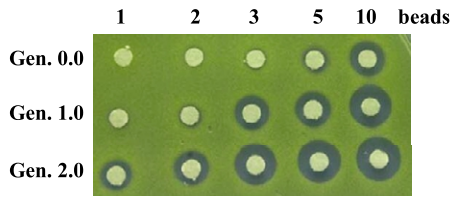


Figure 7. Single and multi-bead screening for compound **D9** on generation 0.0, 1.0, and 2.0 resins.

screening. Quality control of all library members was achieved by LC/MS, and all expected compounds were found with purities between 80 and 100% (λ=254 nm). Only the 3,5-dimethoxyaniline building block did not give the expected compound. Instead, the major compound was found to correspond to the triazine core bearing one R^1 substituent, no R^2 (3,5-dimethoxyaniline) and two R^3 substituents.

The second library daughter plate was prepared for screening by dissolving the library member in DMF and spotting onto discrete 6 mm diameter filter papers, which were then placed on top of 1% agar seeded with *Macrococcus luteus* and incubated at 37°C for 48 h (Fig. 5). Assay revealed the presence of several inhibitors exhibiting zones of inhibition (DMF alone did not affect bacteria growth). Similar activities were obtained with *Bacillus subtilis* bacteria. The top five members of the library chosen for in depth screening and chemical analysis (Fig. 6) were re-synthesised in solution en masse from cyanuric chloride and three, sequential, amine displacements.

Compound **D9**, which gave the best inhibition, was re-synthesised on Generation 1.0 and Generation 2.0 resins. Cleavages from 1, 2, 3, 5, and 10 beads were carried out.[14] Each sample was then screened using the same procedure as described above. The results are shown in Figure 7.

Thus cleavage from a generation 2.0 single bead released enough product to give a clear zone of inhibition. It was found that at least three beads for generation 1.0 and ten beads for generation 0.0 were necessary to give similar zones of inhibition. Single-bead analysis by LC/MS showed that the product was of good purity (Fig. 8).

Zone-based antibacterial assays were successfully developed to screen triazine based antibiotics released from single-beads. Synthesis, based on the attachment of cyanuric chloride onto a Marshall-type safety catch linker, followed by successive aromatic nucleophilic substitutions and linker activation prior to nucleophilic cleavage, was extensively studied and optimised through solution and solid-phase studies. A library of triazines prepared on aminomethyl polystyrene resin allowed the identification of active compounds, which inhibited bacteria growth in a zone-based assay. High-loading dendrimer beads allowed the release of sufficient amount of compound from a single bead to give clear inhibition, which was not the case for non-dendrimerised single beads. In conclusion, the high-loading dendrimerised beads developed in our group continue to act as valuable synthetic supports for single-bead screening and analysis.

2. Experimental

2.1. General

NMR spectra were recorded using a Bruker DPX 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts were reported on the δ scale in ppm and were referenced to residual non-deuterated solvent resonances. Mass spectra were obtained on a VG Platform single quadrupole mass spectrometer in electrospray mode. IR spectra were obtained on a BioRad FTS 135 spectrometer with a Golden gate accessory, with neat compounds as oils or solids with stretches reported in cm^{-1}. Melting points are uncorrected. Commercially available reagents were used without further purification. THF was freshly distilled under nitrogen from a solution of sodium and benzophenone. Purifications by column chromatography were achieved on silica gel 60 (230–400 mesh) purchased from Merck. Analytical HPLC data were obtained using a Hewlett–Packard HP1100 Chemstation, using a Phenomenex C_{18} prodigy 5 μm (150 mm×3 mm) column with a flow rate of 1 ml/min monitoring at the wavelengths of 254 nm and ELS (PL-ELS 1000 from Polymer Laboratories) and eluting with (A) 0.1% TFA in water and (B) 0.042% TFA in acetonitrile, gradient 0% (B) to 100% (B) over 20 min.

LC/MS spectra were obtained on one of the following spectrometers. Micromass ZMD spectrometer in electrospray positive mode using a Phenomenex Synergi 4μ MAX-RP 80A (50 mm×4.60 mm i.d.) column (Method A: from 5 to 95% MeCN in 1% HCOOH in water over 5 min). Waters ZMD spectrometer in electrospray positive mode using a Waters XTerra MS C_{18} 5μ (50 mm×3.0 mm i.d) column (Method B: from 5 to 100% MeOH in 0.1% HCOOH in water over 10 min; Method C: from 5 to 100% MeOH in 0.1% HCOOH in water over 15 min). The preparation of resin-bound dendrimers has been reported elsewhere.[15]

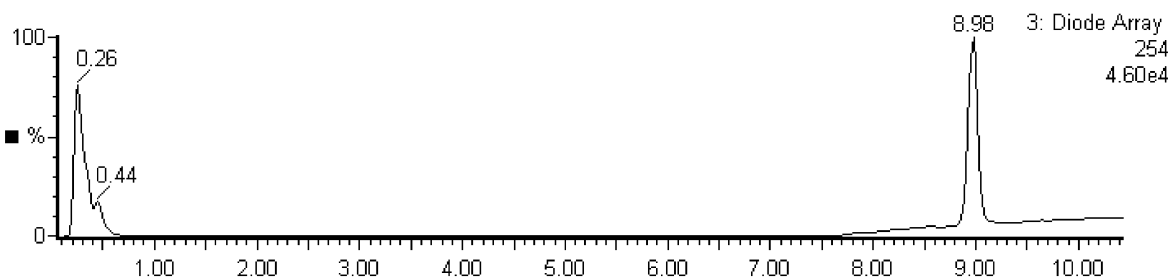


Figure 8. LC/MS analysis from a single-bead cleavage (λ=254 nm).

Inhibition assays. Solutions of each compound in DMF at a concentration between 50 and 1000 μg/ml were spotted onto discrete filter papers (6 mm in diameter) and placed onto seeded agar plates

	Antibacterial dilutions (μg/ml)							
	1000	900	800	700	600	500	400	300
F7	10++	7.5++	8++	8++	8++	7+	7+	
B7	8+	8+	7+					
D9	8++	8++	7.5+	7+	7+			
D5	7.5+	7.5+	7.5+	7.5+	7.5+	7+	7+	
A9	9++	8++	8+	7.5+	7.5+			

Zones of inhibition (mm); ++, clear crisp zone of inhibition; +, non-clear zone of inhibition.

Agar medium was cooled to 45–50°C before the addition of the bacteria (between 10 and 100 μl per 40 ml of LB Agar medium of log phase *M. luteus* or *B. subtilis*). This was shaken gently for a few seconds before pouring into a 130 mm plate. When the medium had hardened completely, the plates were inverted and stored at 4°C.[17]

2.1.1. 2-(4-Hydroxyphenylthio)acetic acid (1). To a stirred solution of 4-mercaptophenol (10 g, 79.25 mmol) in THF (200 ml) was added DIPEA (12 g, 118.8 mmol). The reaction mixture was stirred for 10 min. As the mixture became viscous with the formation of a white precipitate, more THF (100 ml) was added. Bromoacetic acid (16.5 g, 118.8 mmol) was added, and the solution was stirred for 20 h. The solvent was removed in vacuo, and the resulting solid was dissolved in AcOEt (300 ml) and washed with acidified water (2×300 ml, pH 1) and water (2×300 ml). The combined water phases were extracted with AcOEt (1×500 ml). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo to give a yellowish solid. Recrystallisation from AcOEt afforded **1** as a white solid (12.75 g, 69.21 mmol, 87% yield): mp 138°C (lit. 141–142°C);[16] TLC R_f=0.36 (DCM/MeOH/AcOH, 9:1:0.05); ^{1}H NMR (d_6-DMSO) δ 3.67 (s, 2H), 6.85 (d, J=8.5 Hz, 2H), 7.36 (d, J=8.5 Hz, 2H), 9.69 (s, 1H), 12.65 (br s, 1H); ^{13}C NMR (d_6-DMSO) δ 38.2, 116.5, 123.5, 133.3, 157.5, 171.3; IR (neat) ν_{max} 3119, 1686; MS (ES^-) m/z 183.9 (75%) $[M-H]^-$.

2.1.2. 2-[(4-Hydroxyphenyl)thio]-*N*-(phenylmethyl)-acetamide (2a). To a solution of 2-(4-hydroxyphenylthio)-acetic acid **1** (2 g, 10.86 mmol) and HOBt (1.61 g, 11.95 mmol) in DMF (50 ml) was added benzylamine (1.28 g, 11.95 mmol). The reaction mixture was stirred for 5 min before adding DCC (2.7 g, 13.03 mmol). A white precipitate formed within hours. The reaction mixture was stirred for another 17 h. The mixture was filtered and washed with DMF. The filtrate was concentrated in vacuo and then extracted with DCM (250 ml) and water (250 ml). The aqueous phase was extracted with DCM (2×200 ml) and the combined organic phases washed with water (3×200 ml). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. After purification by column chromatography (AcOEt/*iso*hexane, 1:1), **2a** was isolated as a white solid (2.73 g, 9.99 mmol, 92% yield): mp 107–108°C; LC/MS 2.72 min (98%); method A; ^{1}H NMR (d_6-DMSO) δ 3.53 (s, 2H), 4.27 (d, J=6 Hz, 2H), 6.73 (d, J=9 Hz, 2H), 7.15 (d, J=9 Hz, 2H), 7.24–7.31 (m, 5H), 8.45 (t, J=6 Hz, 1H), 9.58 (s, 1H); ^{13}C NMR (d_6-DMSO) δ 39.1, 42.3, 116.0, 123.3, 126.6, 127.1, 128.1, 132.9, 139.1, 157.0, 168.1; IR (neat) ν_{max} 1626, 749, 698; MS (ES^+) m/z 274.4 (100%) $[M+H]^+$, 296.3 $[M+Na]^+$; elemental analysis found C 65.99%, H 5.77%, N 5.78%, S 11.15%; calcd C 65.91%, H 5.53%, N 5.12%, S 11.73%.

2.1.3. 2-[[4-[(4,6-Dichloro-1,3,5-triazin-2-yl)oxy]-phenyl]thio]-*N*-(phenylmethyl)-acetamide (3a). To a solution of cyanuric chloride (177 mg, 0.96 mmol) in THF (2.5 ml) stirred at 0°C was added dropwise a solution of **2a** (250 mg, 0.91 mmol) in THF (0.5 ml) followed by the dropwise addition of a solution of DIPEA (124 mg, 0.96 mmol) in THF (0.5 ml). The resulting mixture was stirred at ice-bath temperature for another hour before warming to room temperature, where it was stirred for 18 h. The reaction mixture was concentrated in vacuo to give an oil which was extracted with DCM (50 ml) and water (50 ml). The organic phase was washed with water (2×50 ml) and the combined aqueous phases were extracted with DCM (2×100 ml). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo to give **3a** as a white solid (379 mg, 0.90 mmol, 98% yield): mp 142–144°C; LC/MS 3.78 min (98%); method A; ^{1}H NMR (d_6-DMSO) δ 3.70 (s, 2H), 4.44 (d, J=6 Hz, 2H), 7.01 (t, J=6 Hz, 1H), 7.10 (d, J=8.5 Hz, 2H), 7.09–7.14 (m, 2H), 7.25–7.27 (m, 3H), 7.33 (d, J=8.5 Hz, 2H); ^{13}C NMR (d_6-DMSO) δ 37.4, 43.8, 122.0, 127.6, 128.7, 129.7, 133.4, 137.6, 149.7, 167.3, 170.9, 173.2; IR (neat) ν_{max} 1640; MS (ES^+) m/z 421.1 (100%) $[M+H]^+$.

2.1.4. 2-[[4-[[4-Chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazin-2-yl]oxy] phenyl] thio]-*N*-(phenylmethyl)-acetamide (4a). To a solution containing **3a** (1.3 g, 3.086 mmol) in THF (30 ml) stirred at 0°C was added a solution of *p*-fluoroaniline (343 mg, 3.086 mmol) in THF (1.5 ml) followed by the addition of a solution of DIPEA (399 mg, 3.086 mmol) in THF (1.5 ml) at such a rate that the temperature always remained below 5°C. After stirring for 2 h at ice-bath temperature, the reaction mixture was concentrated in vacuo to give an oil which was extracted with DCM (100 ml) and water (100 ml). The organic phase was washed with water (2×100 ml) and the combined aqueous phases were extracted with DCM (1×250 ml). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo. The resulting foam was purified by column chromatography (AcOEt/*iso*hexane, 2:3) to give 2-[[4-[[4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazin-2-yl]oxy] phenyl] thio]-*N*-(phenylmethyl)-acetamide as a white foam (1.36 g, 2.75 mmol, 89% yield): mp 86–87°C; LC/MS 3.95 min (98%); method A; ^{1}H NMR (d_6-DMSO) δ 3.78 (s, 2H), 4.31 (d, J=6 Hz, 2H), 7.06 (t, J=9 Hz, 2H), 7.19–7.33 (m, 9H), 7.44–7.51 (m, 2H); ^{13}C NMR (d_6-DMSO) δ 37.0, 42.9, 115.7, 122.8, 123.9, 127.2, 127.6, 128.7, 129.7, 134.0, 134.4, 139.5, 150.4, 158.0, 160.5, 164.9, 170.2, 171.2, 168.3; IR (neat) ν_{max} 1631; MS (ES^+) m/z 496.4 (100%) $[M+H]^+$, 518.5 $[M+Na]^+$.

To a stirred solution of 2-[[4-[[4-chloro-6-[(4-fluorophenyl)-amino]-1,3,5-triazin-2-yl]oxy] phenyl] thio]-*N*-(phenyl-methyl)-acetamide (500 mg, 1.01 mmol) in DMF (10 ml) was added a solution of *p*-anisidine (149 mg, 1.21 mmol) in

DMF (1.0 ml) followed by the addition of a solution of DIPEA (156 mg, 1.21 mmol) in DMF (1.0 ml). The reaction mixture was stirred for 22 h before being concentrated in vacuo to give an oil which was extracted with DCM (80 ml) and water (80 ml). The organic phase was washed with water (3×80 ml) and the combined aqueous phases were extracted with DCM (2×150 ml). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo. Purification by column chromatography (DCM to DCM/MeOH, 95:5) afforded the title compound as a yellowish oil which crystallised within a few hours (584 mg, 1.00 mmol, 99% yield): mp 100°C; LC/MS 4.10 min (100%); method A; ^{1}H NMR (d_6-DMSO) δ 3.69 (s, 2H), 3.79 (s, 3H), 4.45 (d, J=6 Hz, 2H), 6.82 (br d, 2H), 6.94 (br d, 2H), 7.06 (t, J=6 Hz, 1H), 7.10–7.13 (m, 2H), 7.23–7.29 (m, 3H), 7.32 (d, J=7.0 Hz, 2H), 7.34–7.38 (m, 2H), 7.39–7.44 (m, 2H); ^{13}C NMR (d_1-chloroform) δ 38.2, 43.8, 55.5, 114.1, 114.9, 115.3, 122.6, 123.2, 127.6, 128.7, 130.1, 130.8, 134.0, 137.7, 151.4, 156.5, 158.0, 160.4, 165.7, 170.9, 167.7; IR (neat) ν_{max} 1654; MS (ES$^+$) m/z 583.2 (100%) [M+H]$^+$.

2.1.5. 2-[[4-[[4-[(4-Fluorophenyl)amino]-6-[(4-methoxyphenyl)amino]-1,3,5-triazin-2-yl]oxy]phenyl]sulfonyl]-*N*-(phenylmethyl)-acetamide (5a). To a stirred solution of **4a** (50 mg, 0.086 mmol) in DCM (0.5 ml) was added a solution of peracetic acid (39% in AcOH, 43 mg, 0.218 mmol). The reaction mixture was stirred for 7 h before being extracted with DCM (20 ml) and water (20 ml). The organic phase was washed with water (2×20 ml) and the combined aqueous phases were extracted with DCM (1×50 ml). The combined organic phases were dried over $MgSO_4$ and concentrated in vacuo. Purification by column chromatography (AcOEt/*iso*hexane, 3:2) afforded the title compound as a yellowish oil (48.8 mg, 0.079 mmol, 92% yield): LC/MS 4.01 min (100%); method A; ^{1}H NMR (d_6-DMSO) δ 3.72 (s, 3H), 4.15 (s, 2H), 4.32 (d, J=6 Hz, 2H), 6.75 (br d, J=8 Hz, 2H), 6.86–6.90 (m, 2H), 7.20–7.29 (m, 5H), 7.29–7.33 (d, J=9 Hz, 2H), 7.44 (br d, 2H), 7.54 (br d, 2H), 7.87 (d, J=9 Hz, 2H), 8.42 (br t, 1H); ^{13}C NMR (d_1-chloroform) δ 43.6, 55.4, 61.9, 113.7, 114.8, 115.1, 122.7, 123.1, 127.3, 127.8, 128.5, 130.1, 131.7, 135.0, 137.9, 155.9, 157.0, 157.4, 159.8, 161.1, 165.6, 170.1; IR (neat) ν_{max} 1659, 1292, 1151; MS (ES$^+$) m/z 615.2 (100%) [M+H]$^+$.

2.1.6. 4-Hydroxythiophenol resin (2b). To 1% DVB polystyrene aminomethyl resin (250–300 μm, 1 g, 1.38 mmol) was added a solution of 2-(4-hydroxyphenylthio)acetic acid linker (508 mg, 2.76 mmol) and HOBt (372 mg, 2.76 mmol) in DMF (10 ml). After shaking the reaction vessel for 5 min DIC (436 mg, 3.45 mmol) was added. The resin was shaken for 18 h and subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×10 ml each) before being dried in a vacuum oven at 60°C for 24 h. A Ninhydrin test gave a negative result; IR ν_{max} 1625; MAS-NMR ($CDCl_3$) δ 3.48 (br s, 2H); elemental analysis: S 3.64%, which corresponds to 1.14 mmol/g.

2.1.7. 4,6-Dichloro-1,3,5-triazine resin (3b). A suspension of 4-hydroxythiophenol resin **2b** (950 mg, 1.06 mmol) in THF (10 ml) was heated at 50°C under N_2 for 20 min and cooled to 25°C. Cyanuric chloride (902 mg, 4.89 mmol) was then slowly added followed by dropwise addition of a solution of DIPEA (178 mg, 1.38 mmol) in THF (1 ml) at such a rate that temperature never exceeded 25°C. The reaction mixture was shaken for another 20 h and the resin filtered and washed with THF, THF/DCM, and DCM (5×10 ml each). The resin was finally dried in a vacuum oven at 60°C for 24 h to afford 1.12 g of resin; IR ν_{max} 1627; elemental analysis: S 3.18%, which corresponds to 0.99 mmol/g.

2.1.8. [(4-Fluorophenyl)amino]-6-[(4-methoxyphenyl)-amino]-1,3,5-triazine resin (4b). A suspension of resin **3b** (500 mg, 0.515 mmol) in THF (3 ml) was cooled in an ice-bath. A solution of *p*-fluoroaniline (60 mg, 0.541 mmol) in THF (1 ml) was added dropwise. A white precipitate formed which dissolved upon dropwise addition of a solution of DIPEA (70 mg, 0.541 mmol) in THF (0.5 ml). The syringe was left at ice-bath temperature for 1 h with occasional shaking and then shaken at rt for 8 h. The (4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazine resin was subsequently washed with THF, THF/DCM, and DCM (5×3 ml each) before being dried in a vacuum oven at 60°C overnight. 553 mg of resin was obtained; IR ν_{max} 1645; elemental analysis: S 3.07%, which corresponds to 0.96 mmol/g; F 1.69%, which corresponds to 0.89 mmol/g.

To a suspension of 4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazine resin (200 mg, 0.192 mmol) in DMF (1 ml) was added a solution of *p*-anisidine (33 mg, 0.269 mmol) in DMF (0.5 ml) followed a solution of DIPEA (35 mg, 0.269 mmol) in DMF (0.5 ml). The resin was shaken for 18 h, and then subsequently washed with THF, THF/DCM, and DCM (5×2 ml each) before being dried in a vacuum oven at 60°C overnight. 216 mg of resin was obtained; IR ν_{max} 1645; elemental analysis: S 2.73%, which corresponds to 0.85 mmol/g; F 1.53%, which corresponds to 0.81 mmol/g.

2.1.9. [(4-Fluorophenyl)amino]-6-[(4-methoxyphenyl)-amino]-1,3,5-triazine resin (5b). To a suspension of resin **4b** (110 mg, 0.093 mmol) in DCM (1 ml) was added 39% peracetic acid in acetic acid (73 mg, 0.372 mmol). The resin turned from yellow to orange. After shaking for 18 h, the resin was subsequently washed with DCM, DCM/AcOH (1:1), and DCM (5×2 ml each), and dried in vacuum oven at 60°C overnight. 118 mg of resin was obtained; IR ν_{max} 1650, 1284, 1147; elemental analysis: S 2.78%, which corresponds to 0.87 mmol/g; F 1.43%, which corresponds to 0.75 mmol/g.

2.2. Generation 1.0 4-hydroxythiophenol resin (2c)

To generation 1.0 dendrimerised polystyrene resin (250–300 μm, 664 mg, 1.06 mmol) was added a solution of the linker **1** (584 mg, 3.17 mmol) and HOBt (429 mg, 3.17 mmol) in DMF (7 ml). After shaking the reaction vessel for 5 min DIC (534 mg, 4.24 mmol) was added. The resin was shaken for 36 h and subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×10 ml each) before being dried in a vacuum oven at 40°C overnight. A ninhydrin test was negative.

2.3. Generation 1.0 4,6-dichloro-1,3,5-triazine resin (3c)

To a suspension of resin **2c** (255 mg, 0.321 mmol) in THF (1 ml) cooled at 0–5°C under N_2 was slowly added a solution of cyanuric chloride (296 mg, 1.61 mmol) in THF (1.0 ml) followed by the dropwise addition of a solution of DIPEA (166 mg, 1.28 mmol) in THF (1 ml). The resin was left unstirred on an ice-bath temperature for 1 h and then shaken at rt for 22 h. The resin was subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×5 ml each). The resin was finally dried in a vacuum oven at 60°C for 24 h to afford 307 mg of resin; elemental analysis: S 3.46%, which corresponds to 1.08 mmol/g.

2.4. Generation 1.0 [(4-fluorophenyl)amino]-6-[(4-methoxyphenyl)amino]-1,3,5-triazine resin (4c)

To a suspension of resin **3c** (200 mg, 0.216 mmol) in DMF/THF (1:1, 2 ml) at 0–5°C was added a solution of *p*-fluoroaniline (25 mg, 0.224 mmol) in DMF/THF (1:1, 0.3 ml) followed by a solution of DIPEA (29 mg, 0.224 mmol) in DMF/THF (1:1, 0.3 ml). The Generation 1.0 4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazine resin was shaken at rt for 8 h before being subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×5 ml each). After drying the resin in a vacuum oven at 60°C overnight, 200 mg of resin was obtained. To the dry Generation 1.0 4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazine resin (30 mg, 0.03 mmol) was added a solution of *p*-anisidine (15 mg, 0.12 mmol) in DMF (0.3 ml) followed by DIPEA (8 mg, 0.06 mmol). The resin was shaken for 15 h, then heated for 2 h at 50°C, and subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×2 ml each), and finally dried in a vacuum oven at 60°C overnight. 33 mg of resin was obtained.

2.5. Generation 1.0 [(4-fluorophenyl)amino]-6-[(4-methoxyphenyl)amino]-1,3,5-triazine resin (5c)

To a suspension of resin **4c** (33 mg, 0.03 mmol) in DCM (0.3 ml) was added 39% peracetic acid in acetic acid (29 mg, 0.15 mmol). After shaking for 7 h, the resin was subsequently washed with DCM, DCM/AcOH (1:1), and DCM (5×2 ml EACH), and dried in vacuum oven at 60°C overnight. 36 mg of resin was obtained.

2.6. Generation 2.0 4-hydroxythiophenol resin (2d)

To generation 2.0 dendrimerised polystyrene resin (250–300 μm, 300 mg, 0.357 mmol) was added a solution of acid linker **1** (329 mg, 1.79 mmol) and HOBt (241 mg, 1.79 mmol) in DMF (3 ml). After shaking the reaction vessel for 5 min DIC (270 mg, 2.14 mmol) was added. The resin was shaken for 36 h and subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×5 ml each) before being dried in a vacuum oven at 40°C overnight. The reaction was repeated until a satisfactory ninhydrin test was obtained. 397 mg of resin was obtained.

2.7. Generation 2.0 4,6-dichloro-1,3,5-triazine resin (3d)

To a suspension of resin **2d** (50 mg, 0.045 mmol) in THF (1 ml) cooled at −10°C under N_2 was slowly added a solution of cyanuric chloride (415 mg, 2.25 mmol) in THF (2.0 ml) followed by dropwise addition of a solution of DIPEA (79 mg, 0.45 mmol) in THF (1 ml). The resin was left unstirred at ice-bath temperature for 1 h and then shaken at rt for 18 h. The resin was subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×5 ml each). The resin was finally dried in a vacuum oven at 60°C for 24 h to afford 64 mg of resin.

2.8. Generation 2.0 [(4-fluorophenyl)amino]-6-[(4-methoxyphenyl)amino]-1,3,5-triazine resin (4d)

To a suspension of resin **3d** (30 mg, 0.216 mmol) in DMF/THF (1:1, 0.2 ml) at 0–5°C was added a solution of *p*-fluoroaniline (6.2 mg, 0.056 mmol) in DMF/THF (1:1, 0.1 ml) followed by DIPEA (7.3 mg, 0.056 mmol). The resin was shaken at rt for 8 h before being subsequently washed with DMF. DMF/THF, THF, THF/DCM, and DCM (5×2 ml each). After drying the resin in a vacuum oven at 60°C overnight, 200 mg of Generation 2.0 4-chloro-6-[(4-fluorophenyl)amino]-1,3,5-triazine resin was obtained. To a suspension of this resin (0.023 mmol) in DMF (0.2 ml) was added a solution of *p*-anisidine (11 mg, 0.09 mmol) in DMF (0.1 ml) followed by DIPEA (12 mg, 0.09 mmol). The resin was shaken for 36 h plus 2 h at 50°C, then subsequently washed with DMF, DMF/THF, THF, THF/DCM, and DCM (5×2 ml each), and finally dried in a vacuum oven at 60°C overnight.

2.9. Generation 2.0 [(4-fluorophenyl)amino]-6-[(4-methoxyphenyl)amino]-1,3,5-triazine resin (5d)

To a suspension of resin **4d** (0.023 mmol) in DCM (0.2 ml) was added 39% peracetic acid in acetic acid (23 mg, 0.12 mmol). After shaking for 7 h, the resin was subsequently washed with DCM, DCM/AcOH (1:1), and DCM (5×2 ml each), and dried in vacuum oven at 60°C overnight.

2.10. Library synthesis on aminomethyl polystyrene resin

4,6-dichloro-1,3,5-triazine resin **3b** was distributed in a 96-well filter plate (~0.025 mmol/well). After swelling the resin in 0.10 ml of dry THF, solutions of the first set of aniline derivatives in dry THF (0.06 mmol/well) was added followed by DIPEA (0.06 mmol/well). The plate was sealed and shaken on an horizontal shaker for 5 h. The resins were washed with THF and DMF, before adding solutions of the second set of aniline derivatives (0.06 mmol/well) and DIPEA (0.06 mmol/well). The plate was sealed and shaken for 13 h, plus 2 h in a water bath at 55°C. The resins were washed with DMF, THF and DCM before being dried in a vacuum oven at 40°C overnight. The resin was swollen in 0.3 ml DCM. A 39% solution of peracetic acid in acetic acid (0.114 mmol/well) was added and the plate was sealed and shaken for 5 h. The resins were washed with DCM, DCM/AcOH (1:1), and DCM before being dried in a vacuum oven at 40°C overnight. Aliquots of beads (50) were transferred into a 48-well MiniBlock™ where they were swollen in 0.1 ml of DMSO before adding the corresponding solution of cyclic secondary amine (0.06 mmol/well). The plate was sealed and shaken at 100°C for 5 h. The filtrates were collected in a 96-well plate. The resins were washed

thoroughly with DMSO. DMSO and excess of low-boiling point secondary amine removal was carried out on a Genevac™ under vacuum.[14]

2.10.1. Generation 2.0 single-bead cleavage and analysis for D9. LC–MS (ES^+) 8.98 min (100%); method C; HR-MS (ES^+) for $C_{21}H_{25}N_7F$ found 394.2154; calcd 394.2150.

2.10.2. Generation 2.0 single-bead cleavage and analysis for F7. LC–MS (ES^+) 7.35 min (87%); method B; HR-MS (ES^+) for $C_{22}H_{23}N_7O_2F_3$ found 474.1858; calcd 474.1861.

2.10.3. Generation 2.0 single-bead cleavage and analysis for B7. LC–MS (ES^+) 7.32 min (94%); method B; HR-MS (ES^+) for $C_{22}H_{25}N_7OF_3$ found 460.2066; calcd 460.2067.

2.10.4. Generation 2.0 single-bead cleavage and analysis for A9. LC–MS (ES^+) 8.84 min (92%); method B; HR-MS (ES^+) for $C_{20}H_{22}N_6O_2F$ found 397.1780; calcd 397.1783.

2.10.5. Generation 2.0 single-bead cleavage and analysis for D5. LC–MS (ES^+) 7.71 min (94%); method B; HR-MS (ES^+) for $C_{21}H_{23}N_6O_2$ found 391.1874; calcd 391.1877.

2.11. Synthesis of active triazines—general procedure

To a stirred slurry solution of cyanuric chloride (500 mg, 2.71 mmol) in DCM (10 ml) at −10°C was added the first aniline derivative (2.71 mmol) followed by DIPEA (350 mg, 2.71 mmol). The reaction mixture was stirred at −10°C for 1 h. The second aniline (2.71 mmol) was added followed by DIPEA (350 mg, 2.71 mmol). The reaction mixture was stirred for 18 h. The corresponding cyclic amine (2.98 mmol) was added followed by DIPEA (385 mg, 2.98 mmol), the reaction was stirred for 18 h, extracted with DCM (200 ml) and water (200 ml). The aqueous phase was extracted with DCM (2×100 ml) and the combined organic phases were washed with water (2×200 ml) and concentrated in vacuo.

2.11.1. *N*-(3-Fluorophenyl)-6-(4-methyl-piperazin-1-yl)-*N′*-*p*-tolyl-[1,3,5]triazine-2,4-diamine (D9). Purification by column chromatography (DCM/MeOH, 96:4) afforded the title compound as white crystals (851 mg, 2.16 mmol, 80%): mp 186–187°C; LC/MS 6.94 min (100%); method B; ^{1}H NMR (DMSO) δ 2.09 (s, 3H), 2.14 (s, 3H), 2.23 (t, J=4.5 Hz, 4H), 3.64 (t, J=4.5 Hz, 4H), 6.62 (dt, J=2.0, 8.0 Hz, 1H), 6.96 (d, J=8.0 Hz, 2H), 7.14 (q, J=8.0 Hz, 1H), 7.35 (d, J=8.0 Hz, 1H), 7.46 (d, J=8.0 Hz, 2H), 7.69 (d, J=12.0 Hz, 1H), 9.02 (s, 1H), 9.22 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.8, 43.2, 46.2, 54.8, 106.8, 106.9, 108.1, 108.3, 115.7, 120.8, 129.3, 130.1, 130.2, 131.3, 137.8, 142.6, 142.7, 161.5, 163.9, 164.5, 164.9; MS (ES^+) m/z 394.3 (100%) $[M+H]^+$; elemental analysis found C 63.38%, H 6.10%, N 24.61%, F 4.82%; calcd C 64.10%, H 6.15%, N 24.91%, F 4.83%.

2.11.2. *N*-Benzo[1,3]dioxol-5-yl-6-(4-methylpiperazin-1-yl)-*N′*-(4-trifluoromethylphenyl)-[1,3,5]triazine-2,4-diamine (F7). Purification by column chromatography (DCM/MeOH, 96:4) afforded the title compound as a brownish powder (1.09 g, 2.30 mmol, 85%): mp 171–172°C; LC/MS 7.96 min (100%); method B; ^{1}H NMR (DMSO) δ 2.33 (s, 3H), 2.48 (t, J=4.5 Hz, 4H), 3.87 (t, J=4.5 Hz, 4H), 6.09 (s, 2H), 6.95 (d, J=8.5 Hz, 1H), 7.17 (d, J=8.5 Hz, 1H), 7.57 (s, 1H), 7.70 (d, J=8.5 Hz, 2H), 8.07 (d, J=8.5 Hz, 2H), 9.26 (s, 1H), 9.66 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 42.7, 45.6, 54.2, 100.6, 102.5, 107.6, 112.8, 119.1, 121.1, 121.4, 125.4, 134.1, 142.0, 143.9, 146.8, 163.9, 164.3; MS (ES^+) m/z 474.2 (100%) $[M+H]^+$; elemental analysis found C 55.83%, H 4.65%, N 20.56%, F 11.79%; calcd C 55.81%, H 4.68%, N 20.70%, F 12.04%.

2.11.3. *N*-(4-Methoxyphenyl)-6-(4-methylpiperazin-1-yl)-*N′*-(4-trifluoromethylphenyl)-[1,3,5]triazine-2,4-diamine (B7). Trituration with diethylether afforded the title compound as white crystals (1.12 g, 2.44 mmol, 90%): mp 153–156°C; LC/MS 7.34 min (100%); method B; ^{1}H NMR (DMSO) δ 2.33 (s, 3H), 2.47 (t, J=4.5 Hz, 4H), 3.85 (s, 3H), 3.87 (t, J=4.5 Hz, 4H), 7.00 (d, J=4.5 Hz, 2H), 7.70 (d, J=4.5 Hz, 4H), 8.08 (d, J=4.5 Hz, 2H), 9.20 (s, 1H), 9.61 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 43.3, 46.2, 54.9, 55.6, 114.1, 119.6, 121.6, 121.9, 122.5, 126.0, 133.3, 144.6, 155.2, 164.5, 165.0; MS (ES^+) m/z 460.4 (100%) $[M+H]^+$; elemental analysis found C 57.17%, H 4.99%; N 21.03%, F 11.93%; calcd C 57.51%, H 5.26%, N 21.33%, F 12.40%.

2.11.4. *N*-(3-Fluorophenyl)-*N′*-(4-methoxyphenyl)-6-morpholin-4-yl-[1,3,5]triazine-2,4-diamine (A9). Purification by column chromatography (neat DCM) afforded the title compound as an off white powder (657 mg, 1.66 mmol, 61%): mp 161–163°C; LC/MS 8.06 min (100%); method B; ^{1}H NMR (DMSO) δ 3.53 (t, J=4.5 Hz, 4H), 3.61–3.63 (m, 7H), 6.61 (dt, J=2.8, 8.5 Hz, 1H), 6.75 (d, J=9.0 Hz, 2H), 7.13 (q, J=8.0 Hz, 1H), 7.33 (d, J=7.0 Hz, 1H), 7.46 (d, J=9.0 Hz, 2H), 7.68 (d, J=10.5 Hz, 1H), 8.98 (s, 1H), 9.21 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 43.9, 55.6, 66.4, 106.6, 106.9, 108.1, 108.3, 114.1, 115.7, 122.5, 130.1, 130.2, 133.3, 142.6, 142.7, 155.2, 161.5, 163.9, 164.4, 165.1; MS (ES^+) m/z 397.4 (100%) $[M+H]^+$.

2.11.5. *N*-Benzo[1,3]dioxol-5-yl-*N′*-phenyl-6-piperidin-1-yl-[1,3,5]triazine-2,4-diamine (D5). Purification by column chromatography (neat DCM) afforded the title compound as a light yellow powder (853 mg, 2.18 mmol, 81%): mp 135°C; LC/MS 7.49 min (100%); method B; ^{1}H NMR (DMSO) δ 1.63–1.64 (m, 4H), 1.74–1.75 (m, 2H), 3.86 (t, J=5.0 Hz, 4H), 6.08 (s, 2H), 6.92 (d, J=8.5 Hz, 1H), 7.05 (t, J=7.5 Hz, 1H), 7.18 (d, J=8.5 Hz, 1H), 7.37 (t, J=8.0 Hz, 2H), 7.63 (s, 1H), 7.84 (d, J=7.5 Hz, 2H), 9.11 (s, 1H), 9.19 (s, 1H); ^{13}C NMR (DMSO) δ 24.8, 25.9, 44.2, 101.2, 102.9, 108.2, 113.0, 120.3, 122.0, 128.8, 135.2, 140.8, 142.3, 147.4, 146.5, 146.7; MS (ES^+) m/z 391.3 (100%) $[M+H]^+$; elemental analysis found C 64.43%, H 5.76%, N 21.41%; calcd C 64.60%, H 5.68%, N 21.51%.

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