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A Ribozyme with Michaelase Activity: Synthesis of the Substrate Precursors

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Abstract—The ability to generate RNA molecules that can catalyze complex organic transformations not only facilitates the reconstruction and plausibility of possible prebiotic reaction pathways but is also crucial for elucidating the potential of the application of RNA catalysts in organic syntheses. Iterative RNA selection previously identified a ribozyme that catalyzes the Michael addition of a cysteine thiol to an α,β-unsaturated amide. This reaction is chemically similar to the rate limiting step of the thymidylate synthase reaction, which is the corresponding reaction of a cysteine thiol to the double-bond of the uracil nucleobase. Here we provide a detailed description of the synthesis of the ribozyme substrates and the substrate oligonucleotides used for its characterization and the investigation of the background reaction. We also describe the further characterization of the ribozyme with respect to substrate specificity. We show that the thiol group of the cysteine nucleophile is essential for the reaction to proceed. When substituted for a thiomethyl group, no reaction takes place.

Introduction

In vitro selection of complex mixtures of oligoribonucleotides has led to the isolation of an impressive number of new RNA catalysts for a variety of chemical transformations. Examples include the catalysis of acyl transfer reactions,[1,2] self-alkylation reactions,[3,4] RNA ligation,[5] peptide bond formation,[6] cleavage of amide bonds,[7] cleavage of DNA,[8,9] pericyclic reactions[10,11] and isomerization reactions.[12]

We have recently described the isolation of RNA catalysts for a Michael addition of a thiol Michael donor substrate to an α,β-unsaturated carbonyl system, covalently attached to an oligonucleotide substrate.[13] The Michael reaction not only is among the most important in organic synthesis[14] but also plays a role in various cellular processes.[15–17] For example, the rate-limiting step of the reaction sequence catalyzed by thymidylate synthase is the nucleophilic attack of an internal cysteine residue (C198) to the Michael acceptor system of 2′-deoxyuridine-5′-monophosphate (dUMP).[18–20] This step activates dUMP for the subsequent methylation by tetrahydrofolate to yield thymidine monophosphate (TMP). The ribozyme isolated previously by in vitro selection catalyzes a Michael reaction that is chemically analogous to the transformation performed by thymidylate synthase. The selection scheme used for its isolation involved covalent attachment of the α,β-unsaturated Michael acceptor group to the 5′-phosphate of a guanosine-monophosphate residue that served as an initiator nucleotide in an in vitro transcription with T7-RNA polymerase. Thus, every member of the RNA library was selectively derivatized with the Michael acceptor at the 5′-terminus. Upon reaction with a biotinylated cysteine thiol, active catalytic RNAs became labeled with a biotin tag that allowed their immobilization on streptavidin agarose.

Here we provide further details on the design of the in vitro selection scheme, the synthesis and characterization of the required substrates, and the preparation of appropiately modified RNA libraries. We also report

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the background rate of the reaction and the substrate specificity of the ribozyme with respect to the active nucleophilic site of the cysteine in greater detail.

Results and Discussion

Rational design of the selection strategy

The selection scheme was designed so as to allow for the direct selection of catalytically active RNA molecules (Scheme 1). A DNA library of partially randomized sequences with a diversity of approximately 2×10^{15} different molecules consisting of 142 randomized nucleotides served as the template for RNA synthesis by T7 transcription. The RNA library was in vitro transcribed in the presence of fumaramide-derivatized guanosine **1** which served as the initiator nucleotide. Hence, the transcription reaction yielded RNA molecules that contained the fumaramide group covalently attached to their 5′-end. The biotinylated cysteine **5** served as the Michael donor substrate in the selection experiments. Upon formation of the Michael product those RNA sequences that catalyzed the nucleophilic attack of the thiol group (red) of **5** to the double bond of the fumaramide system (blue) became tagged with a biotin anchor. Consequently, active ribozymes became immobilized on streptavidin agarose and could be separated from the non-biotinylated, non-functional RNA species. To achieve the selective removal of the desired RNAs, we had incorporated a photolabile group in the initiator nucleotides. Eluted active sequences were subsequently amplified by reverse transcription, PCR, and in vitro transcription.

Design of substrates

Figure 1 shows the design of the Michael substrates synthesized for the preparation of the RNA selection library. In analogy to the natural Michael donor/acceptor system of the thymidylate synthase, we chose an amide-linked cysteine as the S-nucleophile serving as the Michael donor (**5** in Fig. 1B). This cysteine is covalently bound to biotin via a short diamino alkyl chain and is therefore termed 'biotin cysteine' throughout the text. The biotin serves as an affinity anchor that tags reacted RNA molecules, allowing their selective immobilization out of a large pool of non-reactive RNA species (Scheme 1).

As the Michael acceptor we chose a fumaramide moiety mimicking the Michael acceptor system of the uracile nucleobase in the open chain form. A set of reactions of thiols with different Michael acceptor systems led to the choice of fumaramide as the acceptor substrate, because this moiety exhibited a moderate reactivity in the non-catalyzed background reaction (data not shown). This appeared to be most promising for a successful selection, because highly reactive substrates would apply insufficient selection pressure due to high background reaction rates, whereas unreactive substrates would decrease the chance of finding catalytic species at all. The selection strategy necessitated the covalent attachment of the fumaramide reaction partner to each member of the RNA library. The attachment was achieved by linking it to a guanosine-monophosphate (GMP) (**1**, Fig. 1A) moiety for convenient incorporation into RNA molecules by means of the T7 RNA polymerase (T7 RNAP). It is well established that initiation of enzymatic RNA synthesis can be achieved by chemically modified guanosine monophosphate[21–23] and several examples where substrate-modified GMP-residues are utilized as initiator nucleotides for the preparation of substrate-modified RNA libraries for the selection of catalytic RNAs have been described.[1,10,11] The designed initiator nucleotide was tailored for its application in the current selection process. Thus, the fumaramide group (D) is connected to the GMP (A) via a flexible aminoalkyl-spacer (C) and a photo-cleavable linker (B).[24] The flexible aminoalkyl spacer should give potential catalysts an optimal orientation towards the substrates during the reaction.

The photo-labile unit can be cleaved by irradiation with UV light at 365 nm which allows separation of the RNA from the product after completion of the Michael addition. This feature avoids unintentional enrichment of RNA sequences that become bound to biotin at other potential Michael acceptor sites contained in RNA during the course of selection. *O*-nitrobenzyl groups have been extensively studied as photo-labile protecting groups and linker units in solid phase peptide[25,26] and oligonucleotide synthesis.[27–29] Prior to our study,[13] their suitability and application for in vitro selections of new ribozymes had only been tested in principle in a mock selection.[30] To improve the water solubility of the initiator nucleotide and introduce RNA binding groups into the Michael acceptor, an aminoalkyl unit (E) was also incorporated (Fig. 1).

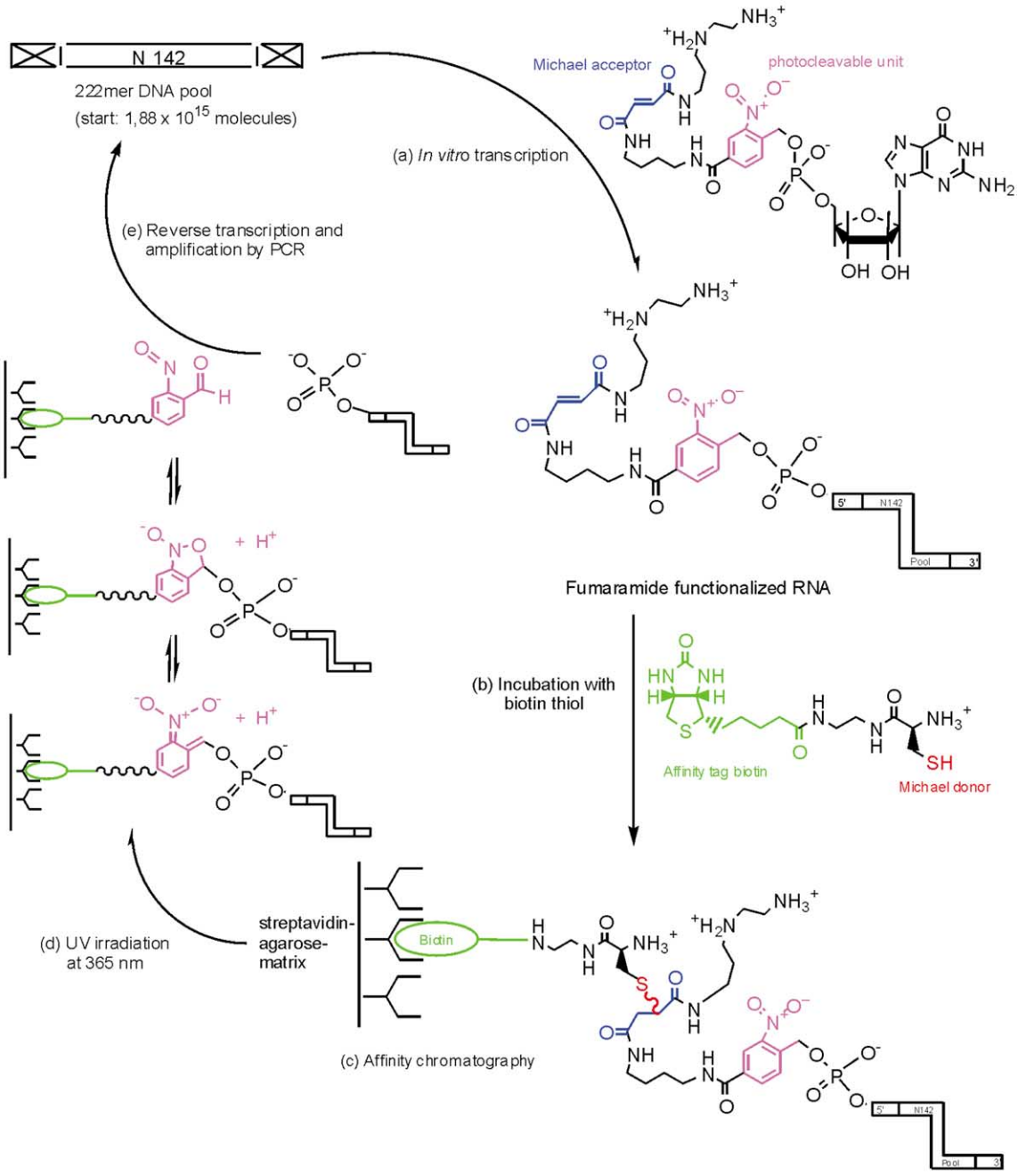
For functional verification of the selected ribozyme activities, it was necessary to synthesize a modified initiator nucleotide **4** which differs from the one used in the selection (**1**) only in having the unsaturated fumaramide unit exchanged by the saturated succinamide unit. The ribozyme molecules modified with **4** cannot be biotinylated by a Michael reaction. This modification was studied to rule out the possibility that the catalytic RNAs were getting attached to the biotin by an unwanted reaction, such as transaminations. In order to further characterize the isolated ribozymes, two more initiator nucleotide derivatives **2** and **3** were synthesized. Both differ from **1** in the substitution of one of the two olefinic hydrogen atoms of the fumaramide unit by a methyl group. Kinetic characterization of ribozymes derivatized with these initiator nucleotides was expected to provide further insights into the reaction mechanism and preferences of the nucleophilic attack on the Michael acceptor system.

As the Michael donor, the free thiol group of biotin cysteine **5** was used in the ribozyme selection. To test whether the thiol group or the free amino of cysteine is the attacking nucleophile, we synthesized compound **6**, where the nucleophilicity of the thiol group is blocked by a methyl group at the sulfur.

Synthesis of the fumaramide-derivatized initiator nucleotides 1–4

The standard procedure for the formation of the phosphate group connecting the guanosine and the photoclevable linker employs the phosphoramidite coupling method.[31–33] This reaction necessitates appropriate protection of the N^2-amino group of the purine ring and the ribose 2′ and 3′- hydroxy groups (Scheme 2A). In the synthesis of the fumaramide-modified guanosine, the N^2-amine of the purine ring was protected as the isobutyryl amide[34] and the 2′- and 3′-hydroxy groups were protected as an acetal group.

N^2-Isobutyryl-2′,3′-isopropylidene-guanosine **8** was prepared from guanosine as illustrated in Scheme 2A. The N^2-amine of guanosine was protected as the isobutyryl amide by the 'transient protection' method developed by Jones and coworkers.[35,36] The resulting N^2-isobutyrylguanosine **7** was converted into the protected guanosine **8** by reaction with 2,2-dimethoxypropane under acidic catalysis. We proceeded to prepare the pho-



Scheme 1. In vitro selection of catalytic RNA for the Michael reaction: (a) in vitro transcription of randomized DNA template with fumaramide-derivatized guanosine generates fumaramide-functionalized RNA libraries; (b) Michael reaction of biotinylated cysteine thiol with the RNA library leads to biotinylation of active RNAs; (c) inactive RNAs are removed by affinity chromatography on streptavidin agarose; (d) active RNAs are isolated after photocleavage; (e) reverse transcription of the active RNAs followed by PCR amplification of the cDNA library affords DNA pool for the next cycle.

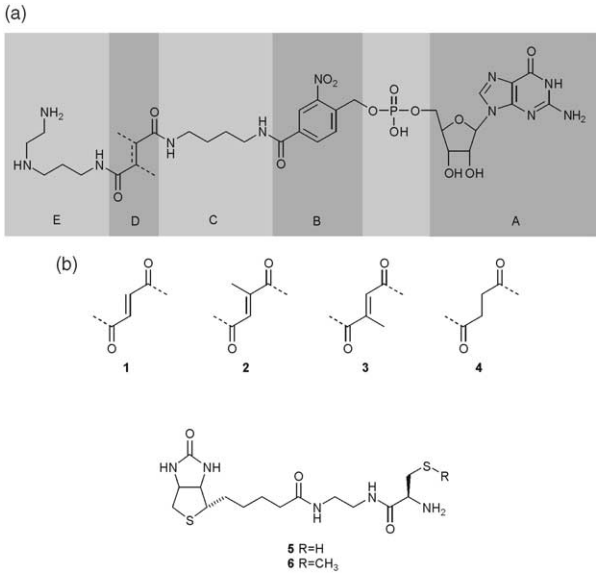


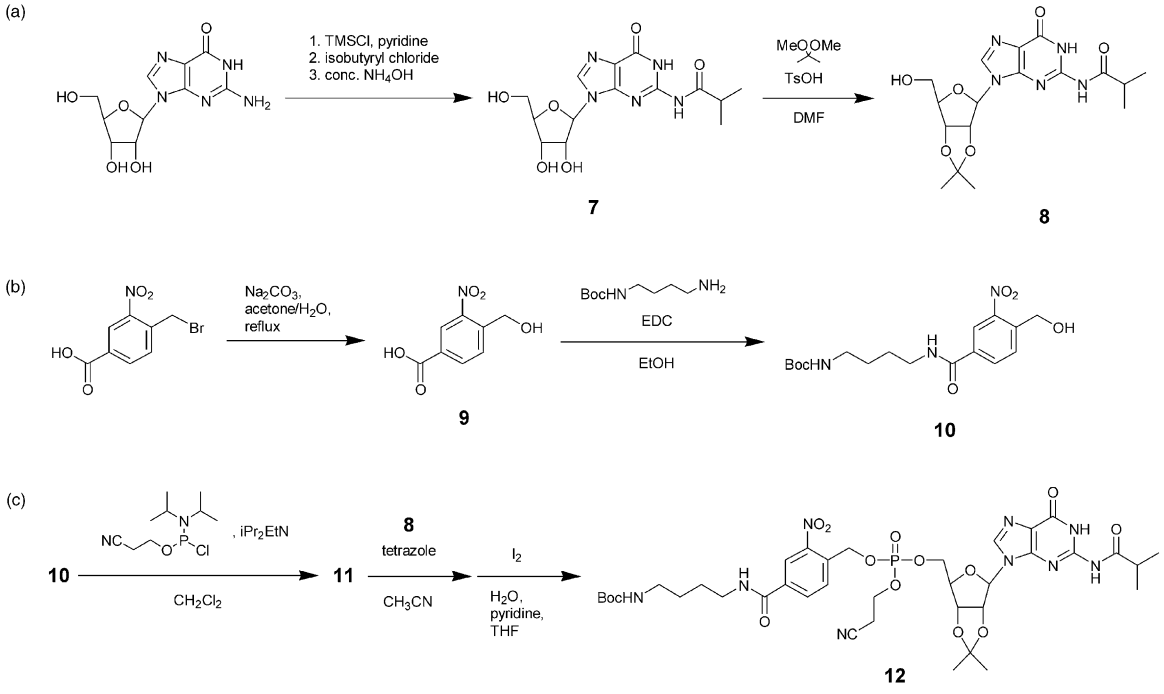
Figure 1. Design of substrates chosen for the isolation and further characterization of catalytic RNAs for the Michael reaction: (A) Fumaramide-derivatized guanosines **1**–**4**; (B) biotinylated cysteine thiols **5** and **6**.

tocleavable unit **10** (Scheme 2B). 4-(Bromomethyl)-3-nitrobenzoic acid[26] was hydrolyzed to give alcohol **9**[37] which was in turn subjected to EDC mediated coupling with *N*-Boc-diaminobutane[38,39] to give alcohol **10**. **10** was subsequently incubated with 2-cyanoethyl *N*,*N*-diisopropyl chlorophosphoramidite to generate the corresponding phosphoramidite **11**. Coupling of the phosphoramidite **11** with guanosine **8**, followed by iodine oxidation, generated the functionalized guanosine **12** (Scheme 2C).

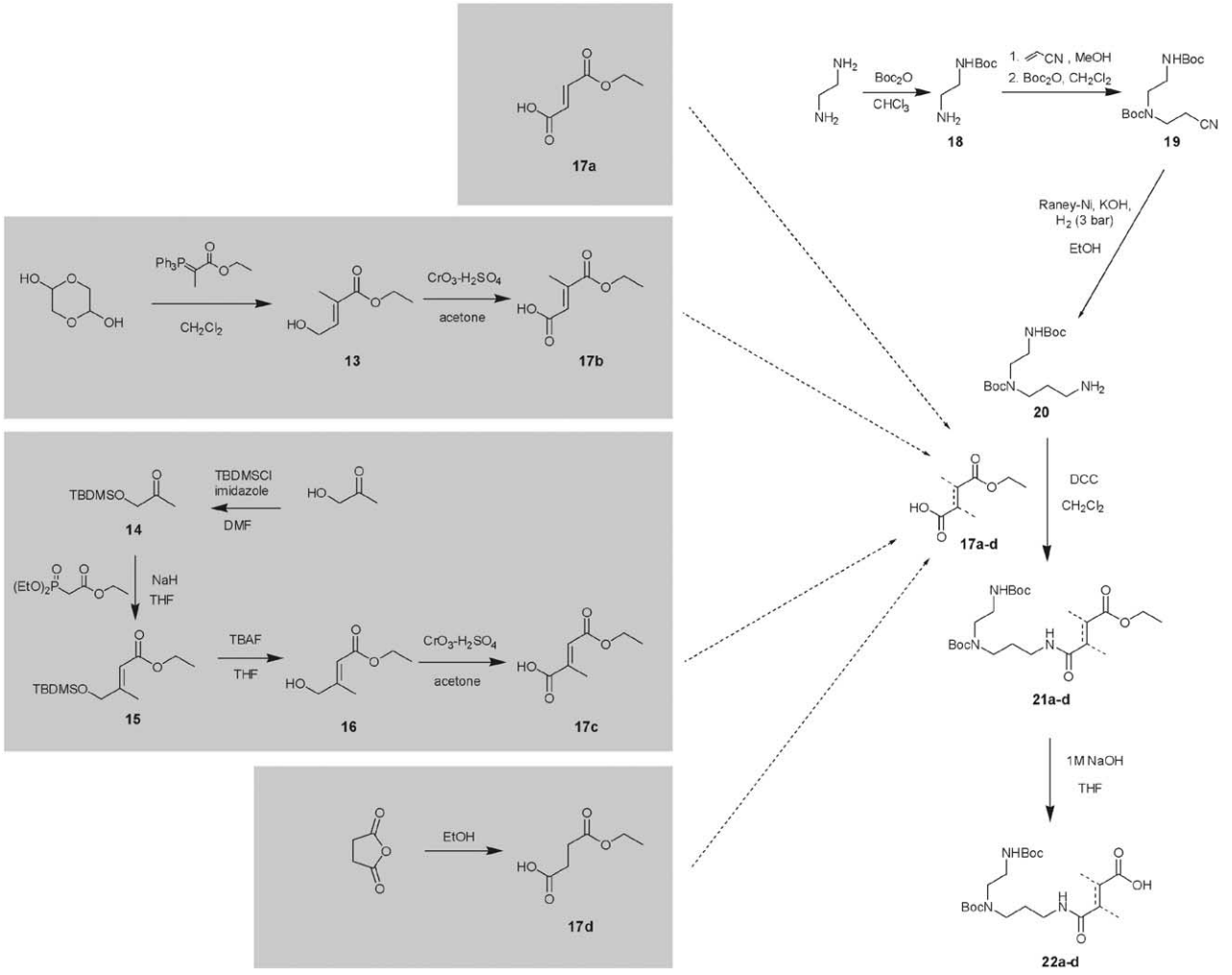
The derivatized fumaric/succinic acids **22a**–**d** were prepared from amine **20** and monoethyl esters **17a**–**d** (Scheme 3). Amine **20** was prepared from *N*-Boc-ethylenediamine in three steps. The Michael reaction of *N*-Boc-ethylenediamine **18**[40] with acrylonitrile, followed by protection of the resulting secondary amine with a Boc group yielded nitrile **19**.[41] Catalytic hydrogenation of this nitrile with Raney nickel gave the partially protected aminoalkyl linker **20**.

While fumaric acid monoethyl ester **17a** is commercially available and succinic acid monoethyl ester **17d** could be obtained in one step from succinic acid anhydride,[42] the methyl substituted fumaric acid monoethyl esters **17b**–**c** were synthesized by Wittig or Horner–Emmons reactions. Ester **17c** was prepared from hydroxypropane. After protection of the hydroxyl group,[43] ketone **14** was subjected to a Horner–Emmons reaction to give ester **15**.[44] Removal of the silyl protecting group and subsequent oxidation of the hydroxyl group using Jones reagent[45] gave the 3-substituted fumaric acid monoester **17c**. The 2-methyl derivative **17b** was synthesized in a similar manner. The corresponding carbonyl compound glycolaldehyde is available in dimerized form, which serves as an intrinsic protection of the hydroxyl group. Thus, glycolaldehyde dimer could be employed without further protection in a Wittig reaction yielding alcohol **13**.[43,46] The latter was oxidized as above and furnished 2-methylfumaric acid monoethyl ester **17b.** Coupling of monoethyl esters **17a-d** to amine **20** with DCC followed by ester cleavage with aqueous NaOH furnished fumaric/succinic acids **22a**–**d**.

Derivatized guanosine **12** was treated with TFA to remove the Boc group, and the resulting amine was



Scheme 2. (A) Synthesis of protected guanosine **8**; (B) synthesis of the photocleavable unit **10**; (C) coupling of guanosine and photocleavable unit to give derivatized guanosine **12**.



Scheme 3. Synthesis of the fumaric acid and succinic acid derivatives **22a**–**d**.

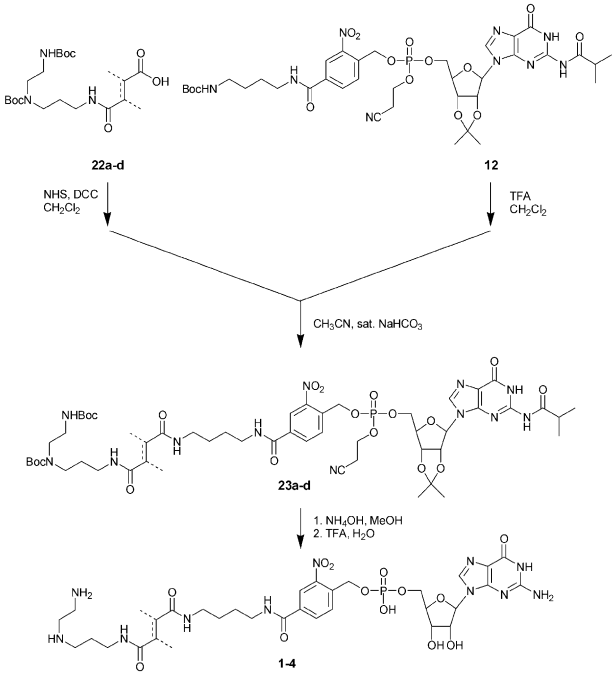
coupled to NHS-activated fumaric and succinic acids **22a**–**d** to generate the protected products **23a**–**d**. Removal of the protecting groups with concentrated NH_4OH followed by aqueous TFA yielded the desired fumaramide derivatized guanosines **1**–**4** (Scheme 4).

Synthesis of the Michael donor substrates 5 and 6

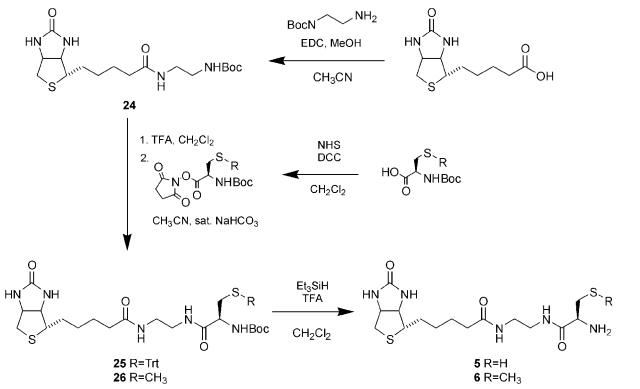
The biotinylated thiols **5**[47] and **6** were prepared as shown in Scheme 5. D-Biotin was coupled to *N*-Boc-ethylenediamine with EDC to obtain carbamate **24**, which was deprotected with TFA and coupled to NHS-activated *N*-Boc-*S*-tritylcysteine or *N*-Boc-*S*-methylcysteine to afford thioethers **25** and **26**, respectively. Triethylsilane and TFA-mediated deprotection of these provided the biotinylated cysteine thiol **5** and its *S*-methylated analogue **6**.[48]

Fumaramide-derivatized RNA libraries

The in vitro selection strategy outlined in Scheme 1 allowed the selection of RNA catalysts for the Michael reaction between a fumaramide group and a thiol group.[13] This selection strategy requires that each member of the RNA library contains a fumaramide



Scheme 4. Synthesis of fumaramide-derivatized guanosines **1**–**4**.



Scheme 5. Synthesis of biotin cysteine thiol **5** and *S*-methylated analogon **6**.

group at its 5′-end. As mentioned earlier, we sought to prepare the fumaramide-modified RNA library by an in vitro transcription reaction in the presence of fumaramide-derivatized initiator nucleotide **1**. To investigate whether the modified guanosines **1**–**4** are accepted by T7 RNA polymerase, these compounds were added to the standard in vitro transcription reactions. The amounts of GTP and **1**–**4** were varied to determine the optimum conditions for the generation of fumaramide-modified RNAs. For these transcription reactions, a 114-mer synthetic DNA template was used which affords 92-mer RNAs.

The RNA molecules produced from the transcription reaction were separated on a 10% denaturing polyacrylamide gel and visualized on a phosphorimager. Figure 2A shows the results of the transcription reaction in the presence of varying amounts of GTP and modified guanosine **1**. The T7 RNA polymerase generates a mixture of the desired RNA (92-mer) and RNA with one additional nucleotide at the 3′-terminus (93-mer).[49] RNA transcripts of 92 (band a) and 93 nucleotides (band b) were generated from the standard transcription reaction (no modified guanosine **1**). As the concentration of **1** was increased, two new bands corresponding to fumaramide-modified 92-mer RNA (band c) and fumaramide-modified 93-mer RNA (band d) were also obtained.

As shown in Figure 2C, maximum amounts (66%) of modified RNA transcripts (compared to total amounts of RNA generated) were obtained when the ratio of GTP/**1** was 1/4. However, at this GTP/**1** ratio the total amount of RNA obtained from the transcription reaction was 30% lower than expected from a standard transcription reaction (data not shown). At ratios of GTP/**1** of 2/3, 3/2, or 4/1, the presence of **1** did not have any detectable inhibitory effect on T7 polymerase activity. Therefore, for further transcription reactions GTP/**1**–**4** ratios of 2/3 were used.

The fumaramide-modified guanosines **1**–**4** contain a photocleavable unit which allows cleavage of the fumaramide group from modified-RNA molecules by irradiation with 365 nm light. We had designed this feature to allow facile removal of active RNAs from the streptavidin matrix during in vitro selection steps. Figure 2B shows that UV irradiation (365 nm, 3 h) allows successful cleavage of fumaramide from the fumaramide-modified RNAs. The cleavage efficiency of the photolabile unit of the initiator nucleotides was determined to

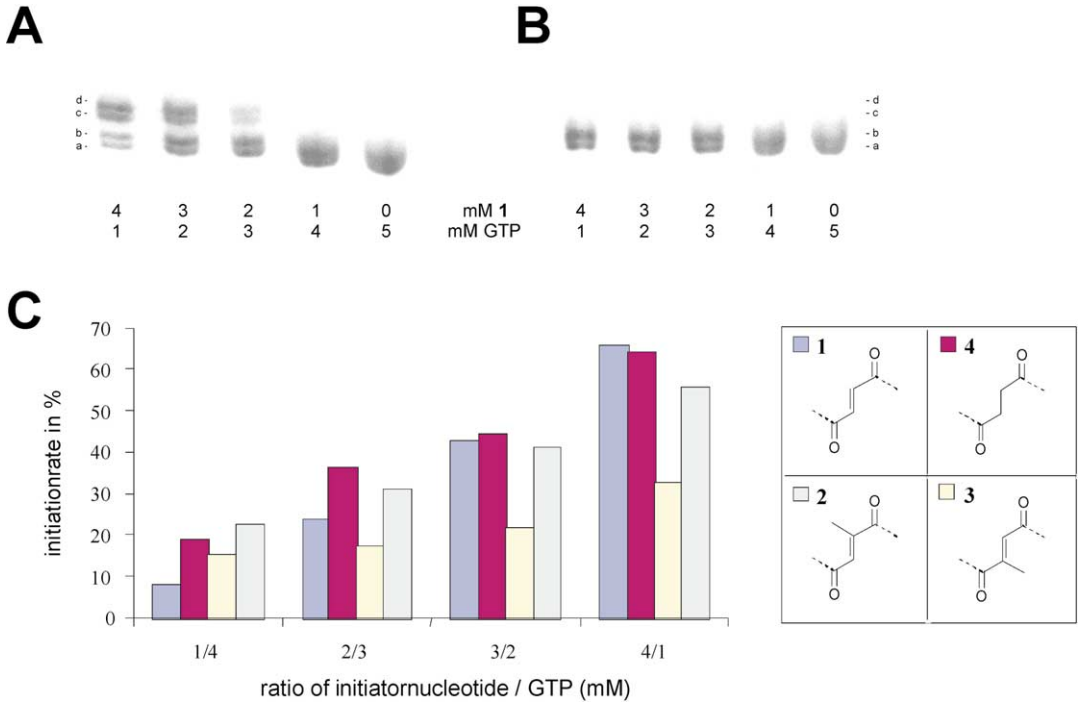


Figure 2. Enzymatic incorporation of initiator nucleotides **1**–**4**. (A) Representative autoradiogram of a transcription reaction of a synthetic 114 bp DNA-template in the presence of initiator nucleotide **1** run by T7-RNA-polymerase. Bands a, b, c, d represent the desired transcripts. Band a: unmodified RNA 92-mer (*n*-mer) transcript, band b: unmodified RNA 93-mer transcript ($n+1$-mer, the additional nucleotide could be A, C, G or U), band c: fumaramide–RNA conjugate (*n*-mer), band d: fumaramide–RNA conjugate ($n+1$-mer). (B) Gelelectrophoretic separation and autoradiography of the same transcripts as in A after exposure to UV light at 365 nm for 3 h. (C) Rate of incorporation for initiator nucleotides **1**–**4**.

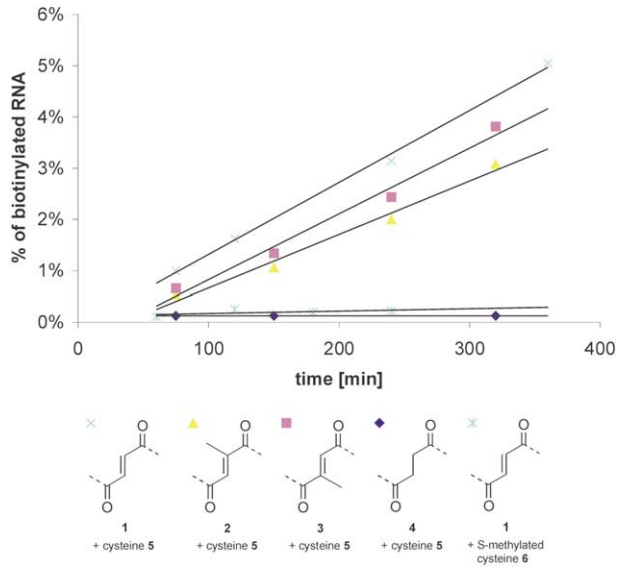


Figure 3. Time course of the background reaction of the Michael reaction between fumaramide derivatized RNA-library (pool-RNA derivatized with **1**–**3**) and biotinylated cysteine **5**. The reactions were carried out under selection conditions [50 mM K-MOPS (pH 7.4), 200 mM NaCl, 5 mM $MgCl_2$, room temperature] at concentrations of [modified RNA] = 1 μM and [biotinylated cysteine] = 30–50 mM. As negative controls, the same experiment was performed using succinamide **4** derivatized pool-RNA or using *S*-methylated cysteine **6**.

be 80–90% in solution, which can be considered to be quantitative. Moreover, as expected, the RNA molecules do not degrade under the cleavage conditions.

Substrate specificity with respect to the nucleophilic Michael donor site

We have reported before that the Michael reaction between fumaramides **1**–**3** and biotin cysteine **5** in the absence of catalyst takes place with a low but measurable rate whereas the succinamide **4** or non-derivatized RNA show no reactivity.[13] Thus, the Michael acceptor system of the fumaramide could be identified as the preferred reaction site for the nucleophilic attack of **5**. In order to also show that the biotinylated cysteine attacks the fumaramide via the thiol group of cysteine, we incubated the fumaramide derivatized RNA library with 30 mM of the *S*-methylated cysteine **6**. The amount of biotinylation detected was negligible (Fig. 3). We also compared the reactions of the fumaramide derivatized ribozyme sequence with cysteines **5** and **6** under the same conditions as in the background reactions. Whereas complete biotinylation of the ribozyme molecules was accomplished by cysteine **5** within 4 h, the rate of biotinylation with the methylated analogue **6** during the same period of time was below 1%. Together, these experiments indicate that the thiol group of the cysteine is indeed the acting nucleophile in the Michael reaction catalyzed by the selected ribozyme.

Conclusion

We have previously reported the selection of a ribozyme for the Michael reaction as an example for RNA catalysts for complex organic reactions with relevance for cellular processes.[13] Here, we describe the design and synthesis of the molecules required for the ribozyme selection experiment and for the characterization of the isolated ribozymes. We also report the incorporation of these molecules into RNA libraries and their role in the underlying selection strategy. Furthermore, we provide details about the investigation of the background reaction and report the results of the further characterization of the ribozyme substrate specificity with respect to the Michael donor group. We have now shown that our ribozyme specifically catalyzes the Michael addition of a cysteine thiol group to an α,β-unsaturated amide. The designed selection scheme utilized a photo-induced ribozyme isolation procedure; we expect this method to be useful in the isolation of other ribozymes as it allows for the separation of active RNA molecules from the mixture in a mild and specific manner.

The in vitro selection methodology, together with the molecules and methods described in this paper, promises to be a generally applicable procedure for the generation of RNA molecules that can catalyze complex organic reactions.

Experimental

Materials and methods

Commercial grade reagents and solvents were used without further purification, except as indicated below. Dichloromethane was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium and benzophenone under nitrogen. Reverse-phase HPLC experiments were conducted with 4.6×250 mm (analytical scale) or 21.4×250 mm (preparative scale) Rainin C_{18} reverse phase columns. All reactions were stirred magnetically in flame- or oven-dried glassware under a positive pressure of Ar or N_2.

***N*²-Isobutyrylguanosine (7).** Guanosine hydrate (10 g, 35.3 mmol) was dried by co-evaporation of its suspension in dry pyridine (3×100 mL) in vacuo. The residue was suspended in dry pyridine (250 mL) under a nitrogen atmosphere, and chlorotrimethylsilane (28.8 g, 265 mmol) was added. The reaction mixture was stirred at ambient temperature for 2 h, cooled to 0 °C, and isobutyryl chloride (11.3 g, 106 mmol) was added dropwise over 20 min. The mixture was allowed to warm to room temperature and stirred for 3 h. The reaction mixture was cooled to 0 °C, and the reaction was quenched by addition of H_2O (30 mL). After stirring for 5 min at 0 °C and then 5 min at room temperature, concentrated aqueous NH_4OH (65 mL) was added. After stirring for an additional 15 min at room temperature, the mixture was diluted with H_2O (500 mL) and washed with CH_2Cl_2 (200 mL). The aqueous layer was concentrated by evaporation in vacuo. The residue was recrystallized from hot H_2O to obtain N^2-isobutyrylguanosine **7** (9.90 g, 79%) as a white solid. ^1H NMR (500 MHz, D_2O): δ 8.10 (s, 1H), 5.90 (d, J = 4.7 Hz, 1H), 4.68 (t, J = 5.0 Hz, 1H), 4.45 (app t, J = 5.1 Hz, 1H), 3.95 (dd, J = 12.7, 3.0

Hz, 1H), 3.86 (dd, $J=12.7$, 4.4 Hz, 1H), 2.77 (app sept, $J=6.9$ Hz, 1H), 1.25 (d, $J=6.8$ Hz, 1H), 1.25 (d, $J=6.8$ Hz, 3H), 1.24 (d, $J=6.8$ Hz, 3H). ^{13}C NMR (125 MHz, D_2O): δ 182.3, 156.8, 149.8, 148.0, 139.6, 119.9, 88.5, 85.1, 74.6, 70.4, 61.5, 36.2, 18.6. IR ($CHCl_3$): 3490–3100, 3165, 3145, 1677, 1604 cm^{-1}. HRMS (FAB): *m/e* for $C_{14}H_{20}N_5O_6$ $[M+H]^+$, calcd 354.1413, found 354.1421.

N^2-Isobutyryl-2′,3′-isopropylidene-guanosine (8). A solution of N^2-isobutyrylguanosine **7** (10 g, 28.3 mmol), 2,2′-dimethoxypropane (200 mL; 1.63 mol), and p-toluenesulfonic acid monohydrate (0.6 g, 3.15 mmol) in DMF (200 mL) was stirred under a nitrogen atmosphere. After 10 h, the reaction mixture was concentrated to dryness and the residue partitioned between equal volumes (100 mL) of CH_2Cl_2 and saturated aqueous $NaHCO_3$. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2×100 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$, 5:95) to afford protected guanosine **8** (9.95 g, 87%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.12 (s, 1H), 8.94 (s, 1H), 7.90 (s, 1H), 5.86 (d, $J=3.9$ Hz, 1H), 5.12 (dd, $J=6.2$, 3.9 Hz, 1H), 5.10–5.08 (m, 1H), 5.00 (dd, $J=6.2$, 2.3 Hz, 1H), 4.41 (app q, $J=2.3$ Hz, 1H), 3.92 (d, $J=12.2$ Hz, 1H), 3.80–3.76 (m, 1H), 2.69 (app sept, $J=6.9$ Hz, 1H), 1.59 (s, 3H), 1.35 (s, 3H), 1.27 (d, $J=7.0$ Hz, 3H), 1.25 (d, $J=7.0$ Hz, 3H). ^{13}C NMR (125 MHz, D_2O): δ 180.1, 155.6, 148.1, 138.7, 129.7, 113.8, 90.5, 86.8, 84.0, 81.1, 62.1, 35.7, 26.9, 24.8, 18.7. IR ($CHCl_3$): 3250–3075, 1695, 1685, 1610 cm^{-1}. HRMS (FAB): *m/e* for $C_{17}H_{24}N_5O_6$ $[M+H]^+$, calcd 394.1726, found 394.1724.

4-(Hydroxymethyl)-3-nitrobenzoic acid (9). A solution of 4-(bromomethyl)-3-nitrobenzoic acid (4.40 g, 16.92 mmol) and Na_2CO_3 (8.96 g, 84.6 mmol) in water/acetone (1:1 v/v, 140 mL) was heated at reflux temperature. After 5 h, the acetone was evaporated. The resulting aqueous solution was washed with ether (80 mL), acidified with half-concentrated HCl and extracted with EtOAc (3×100 mL). The combined organic layers were washed with water (50 mL), dried over $MgSO_4$ and concentrated in vacuo to yield 3.30 g (99%) of 4-(hydroxymethyl)-3-nitrobenzoic acid **9** as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD): δ 8.57 (d, $J=1.7$ Hz, 1H), 8.27 (dd, $J=8.1$, 1.5 Hz, 1H), 7.97 (d, $J=8.1$ Hz, 1H), 4.99 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 167.5 (C-7), 148.4 (C-3), 144.1 (C-4), 135.2 (C-6), 132.1 (C-1), 129.8 (C-5), 126.7 (C-2), 62.0 (C-8). IR (neat): 3595, 1695, 1525, 1315, 1050 cm^{-1}. HRMS (EI): *m/e* for $C_8H_5NO_4$ $[M-H_2O]^+$, calcd 179,0219, found 179,0221.

4-(4-BOC-Aminobutylcarbamoyl)-2-nitrobenzylic alcohol (10). A solution of nitrobenzoic acid **9** (2.72 g, 13.8 mmol), *N*-BOC-diaminobutane (2.60 g, 13.8 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide·HCl (EDC, 3.16 g, 16.5 mmol) in ethanol (100 mL) was stirred for 2 h and then concentrated in vacuo. The residue was dissolved in EtOAc (200 mL), and the solution was washed with 5% aqueous acetic acid (100 mL) followed by saturated aqueous $NaHCO_3$ (50 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated in vacuo to afford 3.5 g of a white solid. Recrystallization from EtOAc/hexanes provided 4-(4-BOC-aminobutylcarbamoyl)-2-nitrobenzylic alcohol **10** (3.0 g, 59%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.41 (s, 1H), 8.02 (d, $J=7.5$ Hz, 1H), 7.80 (d, $J=7.9$ Hz, 1H), 7.59 (br s, 1H); 4.98 (s, 2H), 4.88 (br s, 1H), 3.93 (br s, 1H), 3.47 (app q, $J=5.8$ Hz, 2H), 3.12 (app q, $J=6.5$ Hz, 2H), 1.65 (app quint, $J=6.5$ Hz, 2H), 1.57 (app quint, $J=6.2$ Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.5, 156.5, 147.8, 140.5, 134.6, 132.1, 129.1, 123.4, 79.5, 61.6, 40.1, 39.9, 28.3, 27.9, 25.9. IR ($CHCl_3$): 3477–3150, 3355, 3327, 1683, 1643 cm^{-1}. HRMS (FAB): *m/e* for $C_{17}H_{25}N_3O_6$ $[M+H]^+$, calcd 368.1821, found 368.1819.

[4-(4-BOC-Aminobutylcarbamoyl)-2-nitrobenzyl]-2-cyanoethyl diisopropyl phosphoramidite (11). To a solution of nitrobenzylic alcohol **10** (100 mg, 0.272 mmol) and *N*,*N*′-diisopropylethylamine (105 mg, 0.816 mmol) in CH_2Cl_2 (2 mL) was added at 0 °C 2-cyanoethyl diisopropylchorophosphoramidite (97 mg, 0.408 mmol). After 10 min at 0 °C and a further 20 min at room temperature, the mixture was diluted with EtOAc (75 mL) and washed with saturated aqueous $NaHCO_3$ (25 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (tBME/cyclohexane, 3:1) to afford phosphoramidite **11** (125 mg, 81%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, $J=1.2$ Hz, 1H), 8.10 (dd, $J=8.1$ Hz, $J=1.2$ Hz, 1H), 7.84 (d, $J=8.1$ Hz, 1H), 7.45 (m, 1H), 5.08 (dd, $J=16.2$, 6.9 Hz, 1H), 5.01 (dd, $J=16.1$, 7.75 Hz, 1H), 4.80 (t, $J=5.2$ Hz, 1H), 3.84 (m, 1H), 3.77 (m, 1H), 3.62 (ddt, $J=13.5$, 6.7, 6.8 Hz, 1H), 3.58 (ddt, $J=13.4$, 6.6, 6.7 Hz, 1H), 3.42 (app q, $J=6.1$ Hz, 2H), 3.08 (app q, $J=6.2$ Hz, 2H), 2.59 (t, $J=6.3$ Hz, 2H), 1.60 (app quint, $J=6.8$ Hz, 2H), 1.51 (app quint, $J=6.8$ Hz, 2H), 1.35 (s, 9H), 1,14 (dd, $J=6.6$, 5.4 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.1, 156.5, 146.7, 138.6 (d, $J=7.6$ Hz), 134.7, 132.3, 128.9, 123.5, 117.7, 79.4, 62.4 (d, $J=20$ Hz), 58.5 (d, $J=20$ Hz), 43.3 (d, $J=12.2$ Hz), 40.1, 39.9, 28.4, 28.0, 25.9, 24.6 (d, $J=8$ Hz), 20.4 (d, $J=7$ Hz). ^{31}P NMR (121 MHz, $CDCl_3$): δ 149.1. IR (film): 3335, 2970, 2255, 1690, 1650, 1530, 1365, 1175, 1030, 980 cm^{-1}. MS (FAB): *m/e* 568 ($[M+H]^+$).

N^2-Isobutyryl-2′,3′-isopropyliden-guanosine-5′-monophosphate-P-(2-cyanethyl ester)-P-[4-(4-BOC-aminobutylcarbamoyl)-2-nitro-benzylic ester] (12) (as a mixture of diasteromers). To an ice-cooled solution of protected guanosine **8** (65 mg, 0.164 mmol) and tetrazole (46 mg, 0.656 mmol) in CH_3CN (1 mL) was added phosphoramidite **11** (125 m, 0.220 mmol) in CH_3CN (1.5 mL). The reaction mixture was stirred at 0 °C for 5 min, and then at room temperature for an additional 20 min. The reaction mixture was again cooled to 0 °C, and iodine (1.64 mL, 0.164 mmol, 0.1 M solution in 2:20:80, H_2O/pyridine/THF) was added dropwise over 30 min. The reaction was stirred at 0 °C for 10 min and at room temperature for an additional 15 min. The reaction mixture was concentrated in vacuo, redissolved in 10 mL EtOAc/

2-butanol (7:3) and the solution was washed with aqueous $NaHSO_3$ (0.5 M, 2.5 mL) followed by water (2.5 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5:95) to isolate 100 mg (70%) of modified GMP **12** as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 12.21 (s, 1H), 10.61 (s, 1H), 8.42 (m, 1H), 8.04 (m, 1H), 8.00 (s, 1H), 7.90 (m, 1H), 7.51 (br. s, 1H), 5.92 (m, 1H), 5.39 (m, 2H), 5.23 (m, 1H), 5.12 (br. s, 1H), 4.92 (m, 1H), 4.42 (m, 1H), 4.35 (m, 2H), 4.27 (m, 2H), 3.42 (m, 2H), 3.09 (m, 2H), 2.81 (m, 2H), 2.72 (m, 1H), 1.62 (m, 2H), 1.55 (m, 2H), 1.53 (s, 3H), 1.35 (s, 9H), 1.30 (s, 3H), 1.18 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.3, 165.1, 156.5, 154.9, 148.7, 146.2, 139.1, 136.6, 133.3 (d, $J=9$ Hz), 132.6, 128.0, 124.2, 118.5, 116.3, 114.9, 91.7, 84.9 (d, $J=7$ Hz), 84.0, 81.2, 79.3, 66.5 (d, $J=3$ Hz), 62.8 (d, $J=5$ Hz), 61.7 (d, $J=3$ Hz), 40.1, 36.0, 28.4, 27.7, 27.3, 26.1, 25.5, 19.8 (d, $J=7$ Hz), 19.2. ^{31}P NMR (121 MHz, $CDCl_3$): δ −2.9,−3.0. IR (neat): 3380, 2930, 1685, 1530, 1365, 1255, 1160, 1030 cm^{-1}. HRMS (FAB): *m/e* for $C_{37}H_{51}N_9O_{14}P$ $[M+H]^+$, calcd 876.3293, found 876.3283.

4-Hydroxy-2-methylbut-2-enoic acid ethyl ester (13). To a refluxing solution of glycolaldehyde dimer (790 mg, 6.57 mmol) in CH_2Cl_2 (75 mL) was added dropwise a solution of (1-ethoxycarbonylethyliden)-triphenylphosphorane (5 g, 13.8 mmol) in CH_2Cl_2 (50 mL). After 4 h at refluxing temperature, the solvent was evaporated and the residue purified by flash chromatography on silica gel (ether/petrol ether, 3:1) to give 1.45 g (77%) of γ-hydroxy ester **13** as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 6,75 (tq, $J=6.0$, 1.4 Hz, 1H), 4.27 (dq, $J=6.0$, 0.9 Hz, 2H), 4.12 (q, $J=7,1$ Hz, 2H), 2.75 (s, 1H), 1.76 (app q, $J=1.1$ Hz, 3H), 1.22 (t, $J=7.1$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.9, 140.3, 128.4, 60.8, 59.6, 14.2, 12.6. IR (film): 3360, 2975, 1690, 1365, 1165 cm^{-1}. MS (EI): *m/e* 126 ($[M-H_2O]^+$).

TBDMS-Hydroxypropanone (14). Hydroxypropanone (1.3 mL, 18.6 mmol), TBDMSCl (3.65 g, 24.2 mmol) and imidazole (1.77 g, 26 mmol) were dissolved in DMF (20 mL) and stirred at ambient temperature for 16 h. The mixture was poured onto water (100 mL) and extracted with cyclohexane (3×100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, 8:1) to afford TBDMS-hydroxypropanone **14** (2.91 g, 83%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 4.06 (s, 2H), 2.08 (s, 3H), 0.83 (s, 9H), 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 209.1, 69.5, 25.6, 18.3,−5.5. IR (film): 2930, 2860, 1720, 1255, 1120 cm^{-1}. HRMS (EI): *m/e* for $C_8H_{17}O_2Si$ $[M-CH_3]^+$, calcd 173.0998, found 173.0998.

4-(TBDMS-Hydroxy)-3-methylbut-2-enoic acid ethyl ester (15). Sodium hydride (60% suspension in natural oil, 670 mg, 16.8 mmol) was washed with pentane and then suspended in THF (40 mL). To this suspension was added dropwise with cooling to 20 °C a solution of phosphonoacetic acid triethyl ester (3.10 mL, 15.5 mmol) in THF (8 mL). After stirring at ambient temperature for 1 h until the end of hydrogen formation, TBDMS-hydroxypropanone **14** (2.37 g, 12.58 mmol) was added dropwise with cooling to below 30 °C. After 2 h of stirring at ambient temperature, the mixture was poured onto water (80 mL) and extracted with ether (4×100 mL). The organic combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Purification of the residue by flash chromatography on silica gel (cyclohexane/EtOAc, 20:1) yielded 1.79 g (55%) of γ-hydroxy ester **15** as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 5.90 (m, 1H), 4.09 (q, $J=7.1$ Hz, 2H), 4.02 (m, 2H), 1.96 (m, 3H), 1.20 (t, $J=7.1$ Hz, 3H), 0.84 (s, 9H), 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 157.1, 113.3, 67.1, 59.5, 25.6, 18.3, 15.4, 14.3,−5.5. IR (film): 2930, 2860, 1720, 1220, 1155, 1110 cm^{-1}. HRMS (EI): *m/e* for $C_9H_{17}O_3Si$ $[M-C_4H_9]^+$, calcd 201.0947, found 201.0938.

4-Hydroxy-3-methylbut-2-enoic acid ethyl ester (16). To a solution of 4-(TBDMS-hydroxy)-3-methylbut-2-enoic ethyl ester **15** (1.7 g, 6.58 mmol) in THF (60 mL) was added dropwise at 0 °C a 1 M solution of TBAF in THF (7.3 mL, 7.3 mmol). The mixture was stirred at ambient temperature for 30 min and then concentrated in vacuo. The residue was purified by flash chromatography on silica gel (ether/petrol ether, 3:1) to afford the deprotected γ-hydroxy ester **16** (540 mg, 57%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 5.90 (m, 1H), 4.09 (q, 2H), 4.05 (d, $J=7,1$ Hz, 2H), 2.82 (t, $J=5,8$ Hz, 1H), 2.00 (m, 3H), 1.21 (t, $J=7.1$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.1, 157.6, 113.5, 66.9, 59.8, 15.6, 14.2. IR (film): 3490–3370, 2980, 1715, 1695, 1660, 1225, 1150 cm^{-1}. HRMS (EI): *m/e* for $C_7H_{12}O_3$, calcd 144.0786, found 144.0775.

2-Methylfumaric acid monoethyl ester (17b). To a solution of 4-hydroxy-2-methylbut-2-enoic acid ethyl ester **13** (1.35 g, 9.36 mmol) was added at 0 °C Jones reagent, consisting of chrome(VI)-oxide (2.15 g, 21.53 mmol), water (6,8 mL) and concentrated H_2SO_4 (2 mL). After stirring at ambient temperature for 30 min, EtOH (3.2 mL) and water (20 mL) were added and the mixture was extracted with ether (2×50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. 2-Methylfumaric acid monoethyl ester **17b** was obtained as a colorless oil (1.13 g, 76%). ^1H NMR (400 MHz, $CDCl_3$): δ 10.83 (br. s, 1H), 6.73 (q, $J=1.3$ Hz, 1H), 4.20 (q, $J=7.1$ Hz, 2H), 2.25 (d, $J=1.5$ Hz, 3H), 1.27 (t, $J=7.0$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.4, 166.9, 146.4, 125.7, 61.9, 14.6, 14.1. IR (film): 3100–2900, 1720, 1690, 1645, 1260 cm^{-1}. HRMS (EI): *m/e* for $C_7H_8O_3$ $[M-H_2O]^+$, calcd 140.0473, found 140.0476.

3-Methylfumaric acid monoethyl ester (17c). 4-Hydroxy-2-methylbut-2-enoic acid ethyl ester **16** (500 mg, 3.47 mmol) was oxidized as described above to give 3-methylfumaric acid monoethyl ester **17c** as a colorless oil (540 mg, 98%). ^1H NMR (400 MHz, $CDCl_3$): δ 9.90 (br. s, 1H), 6.82 (q, $J=1.6$ Hz, 1H), 4.17 (q, $J=7.1$ Hz, 2H), 2.21 (d, $J=1.5$ Hz, 3H), 1.25 (t, $J=7.1$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 165.7, 142.5, 128.7, 60.9, 14.1, 13.9. IR (film): 3100–2900, 1720, 1690, 1645, 1285, 1220 cm^{-1}. MS (EI): *m/e* 143 ($[M-CH_3]^+$).

Succinic acid monoethyl ester (17d). Succinic anhydride (50 g, 500 mmol) was dissolved in EtOH (35 mL, 600 mmol) and refluxed for 1 h. Excess EtOH was evaporated to give monoethyl ester **17d** (71.16 g, 97%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.66 (s, 1H), 4.08 (q, J = 7.14 Hz, 2H), 2.61 (t, J = 6.6 Hz, 2H), 2.55 (t, J = 6.3 Hz, 2H), 1.18 (t, J = 7.14 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.0, 172.2, 60.7, 28.8, 28.8, 13.9. IR (film): 2985, 1735, 1170 cm^{-1}. HRMS (EI): m/e for $C_6H_8O_3$ $[M\text{-}H_2O]^+$, calcd 128.0473, found 128.0477.

***N*-BOC-1,2-Diaminoethane (18).** To a solution of 1,2-diaminoethane (28 mL, 400 mmol) in $CHCl_3$ (400 mL) was added dropwise at 0 °C a solution of di-*tert*-butyldicarbonate (8.74 g, 40 mmol) in $CHCl_3$ (200 mL) over a period of 3 h. After stirring at ambient temperature for 16 h, the mixture was washed with brine (6×100 mL) and water (1×100 mL), dried over $MgSO_4$ and concentrated in vacuo to afford 6.40 g (quantitative yield) of *N*-BOC-1,2-diaminoethane **18** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.01 (br. s, 1H), 3.10 (app q, J = 5.7 Hz, 2H), 2.73 (t, J = 6.0 Hz, 2H), 1.38 (s, 9H), 1.12 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.2, 79.1, 43.4, 41.9, 28.4. IR (film): 3500–3100, 2975, 1690, 1525, 1365, 1250, 1170 cm^{-1}.

3-[BOC-(2-BOC-Aminoethyl)-amino]-propionitrile (19). A solution of *N*-BOC-1,2-diaminoethane **18** (10.5 g, 65.54 mmol) and acrylonitrile (6.96 g, 131.08 mmol) in MeOH (150 mL) was stirred for 12 h and then concentrated in vacuo. The residue was redissolved in CH_2Cl_2 (20 mL), di-*tert*-butylcarbonate (16.45 g, 75.37 mmol) was added, and the resulting mixture was stirred at room temperature. After 12 h, the reaction mixture was concentrated in vacuo and chromatographed on silica gel (ether/petrol ether, 3:1) to obtain nitrile **19** (12.90 g, 63%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.89 + 4.73 (br. s, 1H, NH-rotameres), 3.43 (t, J = 6.6 Hz, 2H), 3.33 (t, J = 6.2 Hz, 2H), 3.20 (m, 2H), 2.60 (t, J = 5.3 Hz, 1H), 2.52 (t, J = 5.3 Hz, 1H), 1.41 (s, 9H), 1.37 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.9 + 155.7, 155.0, 118.1 + 117.6, 80.7, 79.2, 47.9 + 46.9, 44.1 + 43.8, 39.1, 28.1, 17.3 + 16.7. IR (neat): 3360, 2980, 1680, 1520, 1165 cm^{-1}. HRMS (EI): m/e for $C_{15}H_{27}N_3O_4$, calcd 313.2002, found 313.2002.

3-[BOC-(2-BOC-Aminoethyl)-amino]-propylamine (20). A solution of nitrile **19** (1.57 g, 5 mmol), Raney Ni[41] (0.4 g) and KOH (0.32 g, 5.75 mmol) in EtOH (40 mL) under H_2 (3.5 bar) was shaken in a parr apparatus. After 5 h, the reaction mixture was filtered over Celite, and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (40 mL) and the solution was washed with saturated aqueous $NaHCO_3$ (20 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to afford 1.58 g (quantitative yield) of amine **20** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.11 + 4.93 (br. s, 1H, NH-rotameres), 3.20 (m, 6H), 2.64 (t, J = 6.5 Hz, 2H), 1.94 (m, 2H), 1.61 (br. s, 2H), 1.40 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.9 + 155.8, 80.0, 79.1, 46.5 + 46.2, 45.2 + 44.4, 39.5, 38.9, 32.5 + 31.3, 28.4. IR (film): 3360, 2975, 1695, 1365, 1165 cm^{-1}. HRMS (EI): m/e for $C_{15}H_{31}N_3O_4$, calcd 317.2315, found 317.2322.

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-acrylic acid ethyl ester (21a). A solution of fumaric acid ethyl ester **17a** (4.09 g, 28.4 mmol), amine **20** (9.00g, 28.4 mmol) and dicyclohexylcarbodiimide (DCC, 7.03 g, 34.1 mmol) in CH_2Cl_2 (100 mL) was stirred at room temperature. After 3 h, the reaction mixture was filtered to remove DCU and the filtrate concentrated in vacuo. The residue was dissolved in EtOAc (200 mL) and the solution washed sequentially with 5% aqueous acetic acid (100 mL) and saturated aqueous $NaHCO_3$ (100 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$, 5:95) to afford 12.2 g (97%) of ester **21a** as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ 7.14–7.02 (br s, 1H), 6.89 (d, J = 15.5 Hz, 1H), 6.74 (d, J = 15.5 Hz, 1H), 4.88 (br s, 1H), 4.22 (q, J = 7.1 Hz, 2H), 3.30–3.23 (m, 8H), 1.72 (app quint, J = 6.3 Hz, 2H), 1.44 (s, 9H), 1.41 (s, 9H), 1.27 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$, 330 K): δ 165.5, 163.8, 156.3, 156.0, 136.7, 129.9, 80.3, 79.4, 60.8, 46.9, 45.6, 39.6, 36.5, 28.3, 27.9, 14.0. IR ($CHCl_3$): 3680–3395, 1641 cm^{-1}. HRMS (FAB): m/e for $C_{21}H_{38}N_7O_7$ $[M + H]^+$, calcd 444.2709, found 444.2710.

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-2-methyl-acrylic acid ethyl ester (21b). 2-Methyl-fumaric acid monoethyl ester **17b** (1.11 g, 7 mmol) was coupled to amine **20** as described above to give 2.16 g (67%) of ester **21b** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.99 + 6.27 (br. s, 1H, NH-rotameres), 6.74 (s, 1H), 5.02 + 4.76 (br. s, 1H, NH-rotameres), 4.17 (q, J = 7.1 Hz, 2H), 3.20 (m, 8H), 2.19 (d, J = 1.2 Hz, 3H), 1.64 (m, 2H), 1.40 (s, 9H), 1.36 (s, 9H), 1.25 (t, J = 7,1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.6, 156.4, 156.0, 138.9, 130.2, 80.4, 79.4, 61.3, 46.6, 43.9, 39.5, 35.6, 30.8, 28.4, 14.2, 13.9. IR (film): 3325, 2975, 1750–1650, 1530, 1415, 1365, 1270, 1170 cm^{-1}. MS (FAB): m/e 458 ($[M + H]^+$).

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-3-methyl-acrylic acid ethyl ester (21c). 3-Methyl-fumaric acid monoethyl ester **17c** (520 mg, 3.3 mmol) was coupled to amine **20** as described above to give 320 mg (21%) of ester **21c** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 + 6.35 (br. s, 1H, NH-rotameres), 6.46 (s, 1H), 5.02 + 4.77 (br. s, 1H, NH-rotameres), 4.14 (q, J = 7.1 Hz, 2H), 3.21 (m, 8H), 2.28 (s, 3H), 1.63 (m, 2H), 1.41 (s, 9H), 1.36 (s, 9H), 1.23 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.3, 166.0, 156.6, 156.0, 143.5, 127.3, 80.7, 79.6, 60.4, 46.5, 43.7, 39.4, 35.9, 30.8, 28.4, 14.2. IR (film): 3335, 2930, 1700, 1670, 1530, 1420, 1370, 1250, 1170. MS (FAB): m/e 458 ($[M + H]^+$), 480 ($[M + Na]^+$).

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-propionic acid ethyl ester (21d). Succinic acid monoethyl ester **17d** (1.56 g, 5 mmol) was coupled to amine **20** as described above to give 1.74 g (80%) of ester **21d** as a colorless oil. ^{1}H NMR (400 MHz,

$CDCl_3$): δ 6.81 + 6.11 (br. s, 1H, NH-rotameres), 5.02 + 4.77 (br. s, 1H, NH-rotameres), 4.07 (q, $J = 7.1$ Hz, 2H), 3.19 (m, 8H), 2.60 (t, $J = 6.5$ Hz, 2H), 2.44 (m, 2H), 1.61 (m, 2H), 1.40 (s, 9H), 1.37 (s, 9H), 1.18 (t, $J = 7{,}1$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.0, 171.7, 156.3, 155.9, 80.3, 79.4, 60.6, 46.6, 43.9, 39.5, 35.9, 31.0, 29.6, 28.4, 27.7, 14.2. IR (film): 3330, 2975, 1750–1650, 1540, 1415, 1365, 1250, 1170 cm^{-1}. MS (FAB): *m/e* 446 ($[M + H]^+$), 468 ($[M + Na]^+$).

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-acrylic acid (22a). A solution of ethyl ester **21a** (4.93 g, 11.1 mmol) in THF (75 mL) was stirred for 1 h with 1 M NaOH (22.3 mL, 22.3 mmol). The reaction mixture was cooled with an ice bath, acidified to pH 2 with concentrated HCl and extracted with $CHCl_3$ (3×100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 3.80 g (82%) of fumaric acid **22a** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ 9.0 (br s, 1H), 7.4 (br s, 1H), 7.18 (d, $J = 15.4$ Hz, 1H), 6.84 (d, $J = 15.5$ Hz, 1H), 5.1 (br s, 1H), 3.36–3.28 (m, 8H), 1.79 (app quint, $J = 6.3$ Hz, 2H), 1.48 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$, 330 K): δ 168.0, 164.2, 156.4, 138.0, 129.7, 80.9, 79.8, 47.0, 45.2, 39.7, 36.9, 28.4, 27.6. IR ($CHCl_3$): 3400–3250, 3320, 1717, 1698, 1670, 1653 cm^{-1}. HRMS (FAB): *m/e* for $C_{19}H_{34}N_3O_7$ $[M + H]^+$, calcd 416.2396, found 416.2396.

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-2-methyl-acrylic acid (22b). Ethyl ester **21b** (2.12 g, 4.63 mmol) was hydrolyzed as described above to give 1.91 g, (96%) of fumaric acid **22b** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (br. s, 1H), 7.34 + 6.87 (br. s, 1H, NH-rotameres), 6.96 (s, 1H), 5.20 + 4.86 (br. s, 1H, NH-rotameres), 3.21 (m, 8H), 2.21 (s, 3H), 1.67 (m, 2H), 1.40 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.5, 166.0, 156.5, 156.0, 138.9, 131.1, 81.0, 79.7, 46.6, 44.4, 39.3, 35.9, 30.8, 28.4, 13.9. IR (neat): 3350, 2975, 1720, 1655, 1515, 1425, 1370, 1250, 1170 cm^{-1}. MS (FAB): *m/e* 430 ($[M + H]^+$).

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-3-methyl-acrylic acid (22c). Ethyl ester **21c** (320 mg, 0.70 mmol) was hydrolyzed as described above to give (300 mg, 99%) of fumaric acid **22c** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (br. s, 1H), 7.51 + 6.32 (br. s, 1H, NH-rotameres), 6.43 (s, 1H), 5.18 + 4.86 (br. s, 1H, NH-rotameres), 3.21 (m, 8H), 2.27 (s, 3H), 1.65 (m, 2H), 1.40 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.1, 168.4, 156.6, 156.0, 149.5, 127.4, 80.9, 79.6, 46.5, 43.9, 39.3, 36.1, 29.7, 28.4, 14.3. IR (neat): 3335, 2930, 1720–1650, 1535, 1420, 1365, 1250, 1170 cm^{-1}. MS (FAB): *m/e* 430 ($[M + H]^+$), 452 ($[M + Na]^+$).

3-{3-[BOC-(2-BOC-Aminoethyl)-amino]-propylcarbamoyl}-propionic acid (22d). Ethyl ester **21d** (1.72 g, 3.85 mmol) was hydrolyzed as described above to give (1.55 g, 96%) of succinic acid **22d** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.05 (br. s, 1H), 7.10 + 6.58 (br. s, 1H, NH-rotameres), 5.18 + 4.86 (br. s, 1H, NH-rotameres), 3.19 (m, 8H), 2.63 (m, 2H), 2.47 (m, 2H), 1.63 (m, 2H), 1.39 (s, 9H), 1.36 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.3, 172.8, 156.3, 156.0, 80.5, 79.8, 46.7, 44.2, 39.3, 36.4, 31.0, 30.0, 28.4, 27.6. IR (neat): 3360, 3140, 2975, 2935, 1720, 1660, 1530, 1420, 1275, 1245, 1180 cm^{-1}. MS (FAB): *m/e* 418 ($[M + H]^+$), 440 ($[M + Na]^+$).

N^2-Isobutyryl-2′,3′-isopropyliden-guanosine-5′-monophosphate-P-(2-cyanethylester)-P-{4-[4-(3-{3-[BOC-(2-BOC-aminoethyl) - amino] - propylcarbamoyl} - acryloylamino)-butylcarbamoyl]-2-nitro-benzylic ester} (23a) (as a mixture of diasteromers). Fumaric acid **22a** (715 mg, 1.72 mmol) was activated by stirring with *N*-hydroxysuccinimide (217 mg, 1.89 mmol) and DCC (389 mg, 1.89 mmol) in CH_2Cl_2 (2 mL) at room temperature. After 30 min, the mixture was filtered and the filtrate was concentrated in vacuo to give the NHS-activated acid as a white solid. At the same time, a solution of modified GMP **12** (1.15 g, 1.32 mmol) and trifluoroacetic acid (TFA, 1.5 mL) in CH_2Cl_2 (15 mL) was stirred under argon. After 30 min, the reaction mixture was concentrated in vacuo and the residue was redissolved in saturated aqueous $NaHCO_3$ (25 mL). The pH of the mixture was adjusted to pH 8 with 1 M NaOH, and a solution of the NHS-activated acid in CH_3CN (25 mL) was added. The reaction mixture was stirred for 1 h and then extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by column chromatography ($MeOH/CH_2Cl_2$, 7:93) to obtain 1.01g (65%) of modified GMP **23a** as a colorless oil. ^{1}H NMR (500 MHz, CD_3OD, 330 K): δ 8.53 (d, $J = 1.7$ Hz, 0.5H), 8.49 (d, $J = 1.7$ Hz, 0.5H), 8.13 (dd, $J = 8.0$, 1.7 Hz, 0.5H), 8.07 (dd, $J = 8.1$, 1.7 Hz, 0.5H), 8.99 (s, 0.5H), 7.98 (s, 0.5H), 7.78 (d, $J = 8.1$ Hz, 0.5H), 7.71 (d, $J = 8.1$ Hz, 0.5H), 6.86 (s, 2H), 6.12 (d, $J = 2.0$ Hz, 0.5H), 6.10 (d, $J = 2.2$ Hz, 0.5H), 5.51 (d, $J = 7.0$ Hz, 0.5H), 5.46 (d, $J = 7.1$ Hz, 0.5H), 5.31 (d, $J = 2.1$ Hz, 0.5H), 5.29 (d, $J = 2.1$ Hz, 0.5H), 5.21 (dd, $J = 6.2$, 3.7 Hz, 0.5H), 5.19 (dd, $J = 6.3$, 3.1 Hz, 0.5H), 4.52–4.45 (m, 2H), 4.39–4.25 (m, 3H), 3.46 (t, $J = 5.9$ Hz, 2H), 3.35 (d, $J = 6.4$ Hz, 2H), 3.29 (app t, $J = 5.0$ Hz, 6H), 3.19 (t, $J = 6.3$ Hz, 2H), 2.91 (t, $J = 6.0$ Hz, 1H), 2.87 (t, $J = 5.8$ Hz, 1H), 2.76 (app sept, $J = 6.9$ Hz, 1H), 1.79 (app quint, $J = 7.0$ Hz, 2H), 1.74–1.68 (m, 4H), 1.58 (s, 1.5H), 1.56 (s, 1.5H), 1.45 (s, 9H), 1.42 (s, 9H), 1.38 (s, 1.5H), 1.36 (s, 1.5H), 1.24–1.21 (m, 6H); ^{13}C NMR (125 MHz, CD_3OD, 330 K): δ 181.7, 181.6, 174.5, 166.9, 166.7, 158.2, 157.4, 157.2, 149.6, 149.5, 148.2, 148.1, 140.3, 140.2, 137.23, 137.20, 135.4 (d, $J = 8.2$ Hz), 135.3 (d, $J = 8.3$ Hz), 133.9, 133.8, 133.43, 133.37, 130.0, 129.9, 124.8, 122.2, 122.1, 118.3 (d, $J = 4.3$ Hz), 115.7 (d, $J = 4.2$ Hz), 92.1, 91.8, 86.7 (d, $J = 7.2$ Hz), 86.5 (d, $J = 7.4$ Hz), 85.7, 85.5, 82.4 (d, $J = 7.7$ Hz), 81.2, 80.1, 69.1, 67.4 (d, $J = 3.7$ Hz), 64.5 (d, $J = 5.4$ Hz), 48.0, 46.5, 40.8, 40.3, 40.2, 38.3, 36.9, 29.1, 28.8, 28.7, 27.7, 27.5, 26.3, 25.7, 20.1 (d, $J = 7.0$ Hz), 19.4, 19.3 (d, $J = 7.7$ Hz); IR (film): 3309, 3178, 1683, 1649 cm^{-1}. MS (FAB): *m/e* for $C_{51}H_{74}N_{12}O_{18}P$ $[M + H]^+$, calcd 1173.5, found 1173.6.

N^2-Isobutyryl-2′,3′-isopropyliden-guanosine-5′-monophosphate-P-(2-cyanethylester)-P-{4-[4-(3-{3-[BOC-(2-BOC-

aminoethyl)-amino]-propylcarbamoyl}-2-methyl-acryloylamino)-butylcarbamoyl]-2-nitro-benzylic ester} (23b) (as a mixture of diasteromers). Fumaric acid **22b** (73 mg, 0.175 mmol) was activated and coupled to modified GMP **12** as described above to give 65 mg (56%) of modified GMP **23b** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52 (m, 1H), 8.04 (s, 1H), 8.02 (m, 1H), 7.71 (m, 1H), 6.46 (m, 1H), 6.12 (m, 1H), 4.99 (m, 2H), 4.88 (m, 1H), 4.60 (m, 1H), 4.48 (m, 1H), 4.34 (m, 2H), 3.44 (m, 2H), 3.31 (m, 4H), 3.26 (m, 4H), 3.17 (m, 2H), 2.91 (m, 1H), 2.82 (m, 2H), 2.18 (m, 3H), 2.03 (m, 2H), 1.74 (m, 2H), 1.68 (m, 2H), 1.45 (s, 9H), 1.41 (s, 9H), 1.28 (s, 6H), 1.24 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 181.8, 168.3, 167.5, 167.1, 158.5, 157.5, 157.3, 147.8, 144.6, 142.6, 140.2, 137.0, 133.7, 132.5, 129.6, 126.3, 125.0, 120.1, 118.5, 115.8, 92.1, 87.1 (d, $J=8$ Hz), 85.6, 82.4, 81.3, 80.1, 69.4 (d, $J=4$ Hz), 67.6 (d, $J=4$ Hz), 64.6 (d, $J=5$ Hz), 50.9, 48.1, 46.1, 40.9, 40.6, 37.7, 37.0, 28.9, 28.8, 27.9, 27.6, 25.7, 20.2 (d, $J=7$ Hz), 19.6, 14.7. ^{31}P NMR (121 MHz, $CDCl_3$): δ −2.3,−2.4. IR (film): 3420–3350, 2925, 2855, 1685, 1530, 1465, 1250, 1030 cm^{-1}. MS (ESI): *m*/*e* 1187 ($[M+H]^+$), 1209 ($[M+Na]^+$).

***N*^{2}-Isobutyryl-2′,3′-isopropyliden-guanosine-5′-monophosphate-P-(2-cyanethylester)-P-{4-[4-(3-{3-[BOC-(2-BOC-aminoethyl)-amino]-propylcarbamoyl}-3-methyl-acryloylamino)-butylcarbamoyl]-2-nitro-benzylic ester} (23c) (as a mixture of diasteromers).** Fumaric acid **22c** (73 mg, 0.175 mmol) was activated and coupled to modified GMP **12** as described above to give 58 mg (43%) of modified GMP **23c** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (d, $J=2$ Hz, 1H), 8.05 (dd, $J=8$, 2 Hz, 1H), 7.92 (s, 1H), 7.88 (d, $J=8$ Hz, 1H), 6.38 (m, 1H), 6.02 (d, $J=2$HZ, 1H), 4.89 (m, 2H), 4.77 (m, 1H), 4.49 (m, 1H), 4.38 (m, 1H), 4.24 (m, 2H), 3.33 (m, 2H), 3.21 (m, 4H), 3.18 (m, 4H), 3.08 (m, 2H), 2.81 (app sept, $J=5$ Hz, 1H), 2.72 (m, 2H), 2.08 (m, 3H), 1.94 (m, 2H), 1.65 (m, 2H), 1.57 (m, 2H), 1.36 (s, 9H), 1.32 (s, 9H), 1.18 (s, 6H), 1.15 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 181.9, 168.4, 167.6, 167.1, 158.5, 157.6, 157.3, 148.5, 144.4, 142.6, 137.0, 135.7, 133.6, 133.0, 129.6, 126.5, 124.6, 120.1, 118.5, 115.6, 92.2, 87.1 (d, $J=7$ Hz), 85.6, 82.4, 81.4, 80.2, 69.3 (d, $J=2$ Hz), 67.7 (d, $J=5$ Hz), 64.6 (d, $J=5$ Hz), 50.9, 48.0, 46.0, 40.8, 40.0, 38.5, 37.0, 28.9, 28.8, 27.9, 27.5, 25.7, 20.2 (d, $J=5$ Hz), 19.5, 14.6. ^{31}P NMR (121 MHz, $CDCl_3$): delta; −2.3,−2.4. IR (film): 3420, 2925, 1655, 1540, 1465, 1365, 1250, 1155 cm^{-1}. MS (FAB): *m*/*e* 1187 ($[M+H]^+$).

***N*^{2}-Isobutyryl-2′,3′-isopropyliden-guanosine-5′-monophosphate-P-(2-cyanethylester)-P-{4-[4-(3-{3-[BOC-(2-BOC-aminoethyl)-amino]-propylcarbamoyl}-propionylamino)-butylcarbamoyl]-2-nitro-benzylic ester} (23d) (as a mixture of diasteromers).** Succinic acid **22d** (73 mg, 0.175 mmol) was activated and coupled to modified GMP **12** as described above to give 52 mg (39%) of modified GMP **23d** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (m, 1H), 7.98 (s, 1H), 7.94 (m, 1H), 7.60 (m, 1H), 6.02 (m, 1H), 4.98 (m, 2H), 4.77 (m, 1H), 4.50 (m, 1H), 4.38 (m, 1H), 4.24 (m, 2H), 3.35 (m, 2H), 3.21 (m, 4H), 3.14 (m, 4H), 3.04 (m, 2H), 2.82 (m, 1H), 2.72 (m, 2H), 2.53 (m, 4H), 1.94 (m, 2H), 1.58 (m, 2H), 1.49 (m, 2H), 1.35 (s, 9H), 1.32 (s, 9H), 1.18 (s, 6H), 1.15 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 181.8, 174.7, 167.1, 158.4, 157.5, 157.3, 147.9, 144.4, 140.2, 136.0 (d, $J=6$ Hz), 133.6, 130.4, 129.6, 125.0, 120.1, 118.5, 115.8, 92.1, 86.7 (d, $J=8$ Hz), 85.6, 82.4, 81.3, 80.1, 69.4 (d, $J=6$ Hz), 67.5 (d, $J=4$ Hz), 64.6 (d, $J=5$ Hz), 50.9, 48.1, 46.1, 40.9, 40.1, 37.9, 37.0, 33.1, 28.8, 28.8, 27.9, 27.6, 25.7, 20.2 (d, $J=7$ Hz), 19.5. ^{31}P NMR (121 MHz, $CDCl_3$): δ −2.3,−2.4. IR (film): 3420–3300, 2925, 1685, 1535, 1365, 1255, 1160, 1035 cm^{-1}. MS (FAB): *m*/*e* 1175 ($[M+H]^+$).

Guanosine-5′-monophosphate-P-[4-(4-{3-[3-(2-aminoethyl-amino)-propylcarbamoyl]-acryloylamino}-butylcarbamoyl)-2-nitro-benzylic ester] (1) (as a mixture of diasteromers). A solution of protected modified GMP **23a** (1.00 g, 0.853 mmol) in MeOH (5 mL) and concentrated NH_4OH (30 mL) was heated at 55 °C in a pressure tube. After 20 h, the reaction mixture was concentrated in vacuo to obtain a yellow oil. The oil was redissolved in H_2O (21 mL) and trifluoroacetic acid (9 mL). The solution was stirred under N_2 for 12 h and then concentrated in vacuo to afford a yellow oil. Purification by preparative HPLC (MeCN/0.15% aqueous TFA, 13:87, 25 mL/min) afforded 0.723 g (82%) of fumaramide modified GMP **1** as a yellow oil. ^{1}H NMR (500 MHz, D_2O): δ 8.88 (s, 1H), 8.29 (d, $J=1.3$ Hz, 1H), 7.93 (d, $J=8.1$ Hz, 1H), 7.74 (d, $J=8.1$ Hz, 1H), 6.70 (d, $J=15.3$ Hz, 1H), 6.62 (d, $J=15.3$ Hz, 1H), 5.87 (d, $J=4.1$ Hz, 1H), 5.10 (t, $J=5.4$ Hz, 2H), 4.70 (t, $J=4.7$ Hz, 1H), 4.39 (t, $J=5.1$ Hz, 1H), 4.31 (s, 1H), 4.17 (s, 2H), 3.35–3.27 (m, 8H), 3.25 (t, $J=5.7$ Hz, 2H), 3.09 (t, $J=7.6$ Hz, 2H), 1.90 (app quint, $J=7.4$ Hz, 2H), 1.59 (br s, 4H). ^{13}C NMR (125 MHz, D_2O): δ 167.4, 167.2, 166.6, 163.0 (q, $J=36$ Hz), 155.5, 154.9, 149.7, 146.1, 137.3 (d, $J=8.9$ Hz), 136.4, 134.4, 133.0, 132.8, 132.1, 128.9, 123.9, 116 .5 (q, $J=290$ Hz), 108.5, 90.4, 84.7 (d, $J=8.1$ Hz), 73.9, 70.0, 65.4 (d, $J=5.2$ Hz), 64.5, 46.0, 44.6, 40.1, 39.7, 36.7, 35.8. 26.0, 25.9, 25.8. IR (film): 3554–2593, 3307, 3079, 2941, 1675, 1639 cm^{-1}. MS (ESI): *m*/*e* for $C_{31}H_{45}N_{11}O_{13}P$ $[M+H]^+$, calcd 810, found 810.

Guanosine-5′-monophosphate-P-[4-(4-{3-[3-(2-aminoethyl-amino)-propylcarbamoyl]-2-methyl-acryloylamino}-butylcarbamoyl)-2-nitro-benzylic ester] (2) (as a mixture of diasteromers). Modified GMP **23b** (40 mg, 0.034 mmol) was deprotected as described above and the product purified by MPLC (MeCN/0.15% aqueous TFA, 8:92) to give 6 mg (21%) of fumaramide modified GMP **2** as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.88 (br. s, 1H), 8.32 (s, 1H), 7.95 (d, $J=8$ Hz, 1H), 7.75 (d, $J=8$ Hz, 1H), 6.28 (s, 1H), 5.88 (d, $J=4$ Hz, 1H), 5.10 (app t, $J=5$ Hz, 2H), 4.71 (app t, $J=5$ Hz, 1H), 4.38 (app t, $J=5$ Hz, 1H), 4.31 (m, 1H), 4.15 (app t, $J=3$ Hz, 2H), 3.41 (t, $J=7$ Hz, 2H), 3.33 (m, 4H), 3.27 (m, 4H), 3.08 (t, $J=8$ Hz, 2H), 2.02 (s, 3H), 1.88 (app quint, $J=7$ Hz, 2H), 1.59 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.8, 171.4, 157.7, 148.3, 145.5, 139.7 (d, $J=9$ Hz), 136.7, 135.1, 131.2, 127.4, 126.2, 120.4, 92.6, 87.0 (d, $J=8$ Hz), 76.0, 72.3, 67.7 (d, $J=5$ Hz), 66.7 (d, $J=3$ Hz), 53.0, 48.3, 46.8, 42.4, 42.0, 38.6, 38.0, 28.3, 28.2, 16.6. ^{31}P NMR (121 MHz, $CDCl_3$): δ −0.8. IR (film):

3420, 1680, 1640, 1535, 1205 cm^{-1}. MS (FAB): *m/e* 824 ($[M+H]^+$), 846 ($[M+Na]^+$).

Guanosine-5′-monophosphate-P-[4-(4-{3-[3-(2-aminoethylamino)-propylcarbamoyl]-3-methyl-acryloylamino}-butylcarbamoyl)-2-nitro-benzylic ester] (3) (as a mixture of diasteromers). Modifed GMP **23c** (58 mg, 0.049 mmol) was deprotected as described above and the product purified by MPLC (MeCN/0.15% aqueous TFA, 8:92) to give 9 mg (22%) of fumaramide modified GMP 3 as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ 8.79 (br. s, 1H), 8.47 (s, 1H), 8.08 (d, $J=8$ Hz, 1H), 7.87 (d, $J=7$ Hz, 1H), 6.52 (s, 1H), 5.97 (d, $J=5$ Hz, 1H), 5.23 (app t, $J=6$ Hz, 2H), 4.84 (m, 1H), 4.52 (app t, $J=5$ Hz), 4.43 (m, 1H), 4.28 (m, 2H), 3.47 (m, 6H), 3.38 (m, 4H), 3.20 (t, $J=8$ Hz, 2H), 2.09 (s, 3H), 2.02 (m, 2H), 1.73 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.2, 174.0, 171.0, 159.3, 148.4, 148.1, 144.7, 143.4, 140.0 (d, $J=9$ Hz), 136.6, 135.1, 131.1, 130.0, 128.8, 126.1, 120.5, 91.9, 86.9 (d, $J=8$ Hz), 75.8, 72.5, 67.9 (d, $J=5$ Hz), 66.7 (d, $J=2$ Hz), 53.0, 48.3, 46.8, 42.4, 41.6, 39.0, 38.0, 28.4, 28.2, 16.4. ^{31}P NMR (121 MHz, $CDCl_3$): δ −0.8. IR (film): 3425, 1685, 1640, 1540, 1205 cm^{-1}. MS (FAB): *m/e* 824 ($[M+H]^+$), 846 ($[M+Na]^+$).

Guanosine-5′-monophosphate-P-[4-(4-{3-[3-(2-aminoethylamino) - propylcarbamoyl] - propionylamino} - butylcarbamoyl)-2-nitro-benzylic ester] (4) (as a mixture of diasteromers). Modifed GMP **23d** (52 mg, 0.044 mmol) was deprotected as described above and the product purified by MPLC (MeCN/0.15% aqueous TFA, 8:92) to give 7 mg (20%) of fumaramide modified GMP **4** as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ 8.70 (br. s, 1H), 8.38 (s, 1H), 7.98 (d, $J=8$ Hz, 1H), 7.80 (d, $J=8$ Hz, 1H), 5.87 (m, 1H), 5.12 (app t, $J=6$ Hz, 2H), 4.73 (m, 1H), 4.42 (app t, $J=5$ Hz), 4.32 (m, 1H), 4.18 (m, 2H), 3.38 (m, 2H), 3.34 (m, 4H), 3.17 (m, 4H), 3.05 (t, $J=8$ Hz, 2H), 2.45 (m, 4H), 1.82 (app quint, $J=8$ Hz, 2H), 1.57 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.9, 177.2, 170.0, 157.7, 148.3, 146.0, 139.8 (d, $J=7$ Hz), 136.7, 135.1, 132.0, 131.2, 126.2, 120.4, 92.6, 87.0 (d, $J=9$ Hz), 76.0, 72.3, 67.6 (d, $J=5$ Hz), 66.7 (d, $J=4$ Hz), 53.0, 48.2, 46.8, 42.4, 41.6, 38.5, 38.0, 33.7, 33.6, 28.5, 28.3. ^{31}P NMR (121 MHz, $CDCl_3$): δ −0.8. IR (film): 3315, 1675, 1640, 1535, 1385, 1200 cm^{-1}. MS (ESI): *m/e* 812 ($[M+H]^+$), 834 ($[M+Na]^+$).

***N*-BOC-*N*-[2-(D-Biotinylamino)-ethyl]-amin (24).** A solution of D-biotin (1000 mg, 4.09 mmol), *N*-BOC-1,2-diaminoethane **18** (787 mg, 4.91 mmol) and EDC (1020 mg, 5.32 mmol) in MeOH (10 mL) and CH_3CN (30 mL) was stirred for 5 h at room temperature and then concentrated in vacuo. The residue was resuspended in MeOH and filtrated over Celite. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 90:10) to give 882 mg (56%) of protected amine **24** as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 4.93 (dd, $J=7.9$, 4.4 Hz, 1H), 4.21 (dd, $J=7.9$, 4.4 Hz, 1H), 3.13 (q, $J=6.1$ Hz, 2H), 3.10 (dd, $J=5.4$, 4.9 Hz, 1H), 3.04 (t, $J=5.8$ Hz, 2H), 2.82 (dd, $J=12.7$, 5 Hz, 1H), 2.60 (d, $J=12.8$ Hz, 1H), 2.10 (t, $J=7.5$ Hz, 2H), 1.68–1.45 (m, 4H), 1.33 (s, 9H), 1.19 (s, 2H). ^{13}C NMR: (100 MHz, $CDCl_3$) δ 176.4, 166.2, 158.6, 80.2, 63.4, 61.7, 57.0, 41.1, 40.5, 36.9, 29.8, 29.5, 28.8, 26.8. IR (film): 3300, 2935, 1695, 1650, 1535, 1265, 1175 cm^{-1}. HRMS (FAB): *m/e* for $C_{17}H_{31}N_4O_4S$ $[M+H]^+$, calcd 387.2066, found 387.2066.

***N*-[2-(D-Biotinylamino)-ethyl]-(*N*-BOC-*S*-trityl-L-cysteine)-amide (25).** A solution of *N*-BOC-*S*-trityl-L-cysteine (428 mg, 0.92 mmol), NHS (117 mg,1.02 mmol) and DCC (210 mg, 1.02 mmol) in CH_2Cl_2 (2 mL) was stirred for 30 min at room temperature and then filtered and concentrated in vacuo. The residue was recrystallized from iPrOH to give the NHS-ester. A solution of protected amine **24** (238 mg, 0.62 mmol) and trifluoroacetic acid (0.71 mL, 9.24 mmol) in CH_2Cl_2 (6 mL) was stirred for 1 h at room temperature and then concentrated in vacuo. The residue was redissolved in saturated aqueous $NaHCO_3$ (8 mL) and a solution of the NHS-ester in CH_3CN (6 mL) was added. The mixture was stirred for 10 h and then extracted with EtOAc (3×30 mL). The organic layers were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 90:10) to give 379 mg (84%) of biotin modified cysteine **25** as a white solid. ^1H NMR (500 MHz, $CDCl_3$): δ 7.39 (dd, $J=7.5$, 1.2 Hz, 6H), 7.28 (t, $J=7.3$ Hz, 6H), 7.21 (t, $J=7.2$ Hz, 3H), 7.05 (br s, 2H), 6.93 (br s, 1H), 6.19 (br s, 1H), 5.23 (br s, 1H), 4.42 (dd, $J=7.6$, 4.9 Hz, 1H), 4.24 (dd, $J=7.5$, 4.6 Hz, 1H), 3.90 (app q, $J=5.3$ Hz, 1H), 3.39–3.32 (m, 2H), 3.27–3.19 (m, 2H), 3.11–3.07 (m, 1H), 2.82 (dd, $J=12.8$, 4.7 Hz, 1H), 2.68 (d, $J=12.7$ Hz, 1H), 2.61 (dd, $J=12.6$, 7.1 Hz, 1H), 2.53 (dd, $J=12.6$, 5.6 Hz, 1H), 2.25 (br s, 1H), 2.08 (t, $J=7.2$ Hz, 2H), 1.75–1.53 (m, 4H), 1.40 (s, 9H), 1.40–1.27 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.9, 171.4, 164.5, 155.4, 144.7, 144.3, 129.5, 128.0, 127.9, 126.9, 80.1, 67.1, 61.7, 60.3, 55.7, 53.7, 40.4, 39.6, 39.1, 35.7, 34.1, 28.3, 28.1, 28.0, 25.3. IR ($CHCl_3$): 3520–3170, 3305, 1690 cm^{-1}. HRMS (FAB): *m/e* for $C_{39}H_{50}N_5O_5S_2$ $[M+H]^+$, calcd 732.3253, found 732.3252.

***N*-[2-(D-Biotinylamino)-ethyl]-(*N*-BOC-*S*-methyl-L-cysteine)-amide (26).** *N*-BOC-*S*-methyl-L-cysteine (217 mg, 0.92 mmol) was coupled to protected amine **24** as described above to afford 343 mg (99%) of biotin modified *S*-methylcysteine **26** as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 4.43 (dd, $J=7.8$, 4.8 Hz, 1H), 4.24 (dd, $J=7.8$, 4.3 Hz, 1H), 4.08 (dd, $J=7.5$, 6.0 Hz, 2H), 3.21 (m, 4H), 3.13 (ddd, $J=8.4$, 5.5, 4.9 Hz, 1H), 2.85 (dd, $J=12.8$, 4.9 Hz, 1H), 2.78 (dd, $J=13.8$, 5.7, 1H), 2.61 (d, $J=12.8$ Hz, 1H), 2.59 (d, $J=13.8$ Hz, 1H), 2.11 (t, $J=7.3$ Hz, 2H), 2.03 (s, 3H), 1.69–1.45 (m, 2H), 1.55 (quint, $J=6.8$ Hz, 2H), 1.36 (s, 9H), 1.19 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.5, 174.2, 166.0, 157.9, 81.0, 63.4, 61.8, 57.0, 55.6, 41.1, 40.1, 37.4, 36.9, 29.8, 29.5, 28.8, 26.8, 15.8. IR (film): 3650–3200, 1685 cm^{-1}.

***N*-[2-(D-Biotinylamino)-ethyl]-L-cysteine-amide (5).** A solution of protected cysteine **25** (260 mg, 0.329 mmol), triethylsilane (77 mg, 0.658 mmol), and trifluoroacetic acid (5 mL, 65.8 mmol) in CH_2Cl_2 (5 mL) was stirred under a nitrogen atmosphere. After 20 min, the reaction

mixture was concentrated in vacuo and redissolved in a mixture of H_2O (5 mL) and CH_2Cl_2 (5 mL). The layers were separated and the aqueous layer was washed with CH_2Cl_2 (2×15 mL). The aqueous layer was concentrated in vacuo to afford 160 mg (97%) of biotinylated cysteine **5** as a colorless oil. ^{1}H NMR (500 MHz, D_2O): δ 4.42 (app t, J = 6.0 Hz, 1H), 4.24 (dd, J = 6.5, 4.5 Hz, 1H), 3.97 (app t, J = 5.6 Hz, 1H), 3.29 (app t, J = 6.9 Hz, 1H), 3.17–3.12 (m, 4H), 2.90–2.60 (m, 4H), 2.08 (t, J = 7.1 Hz, 2H), 1.56–1.35 (m, 4H), 1.21 (q, J = 6.9 Hz, 2H). ^{13}C NMR (125 MHz, D_2O): δ 177.4, 168.3, 165.5, 62.5, 60.7, 55.7, 54.9, 40.0, 39.3, 38.7, 35.8, 28.3, 28.0, 25.4, 25.2. IR (film): 3410–3200, 3295, 1675 cm^{-1}. HRMS (FAB): m/e for $C_{15}H_{28}N_5O_3S_2$ $[M+H]^+$, calcd 390.1633, found 390.1630.

***N*-[2-(D-Biotinylamino)-ethyl]-(*S*-methyl-L-cysteine)-amide (6).** Methylcysteine **26** (50 mg, 0.1 mmol) was deprotected as described above to give 40 mg (98%) of biotinylated *S*-methylcysteine **6** as a colorless oil. ^{1}H NMR (400 MHz, D_2O): δ 4.45 (dd, J = 7.9, 4.4 Hz, 1H), 4.27 (dd, J = 7.9, 4.4 Hz, 1H), 3.99 (dd, J = 7.9, 5.7 Hz, 1H), 3.32 (td, J = 8.9, 4.1 Hz, 1H), 3.22–3.15 (m, 4H), 2.90–2.60 (m, 4H), 2.11 (t, J = 7.3 Hz, 2H), 2.00 (s, 3H), 1.62–1.37 (m, 2H), 1.51 (q, J = 7.3 Hz, 2H), 1.26 (q, J = 7.5 Hz, 2H). ^{13}C NMR (100 MHz, D_2O): δ 179.8, 171.2, 167.2, 64.7, 62.9, 57.9, 54.4, 42.3, 40.8, 38.1, 37.0, 30.6, 30.3, 27.7, 17.2. IR (film): 3600–3100, 1680 cm^{-1}. MS (FAB): m/e 404 ($[M+H]^+$), 426 ($[M+Na]^+$).

Kinetic study of background Michael reactions by affinity chromatography

The ^{32}P-labeled RNA library derivatized with one of the compounds **1**–**4** was dissolved in aqueous buffer (50 mM K-MOPS, 200 mM NaCl, pH 7.4) to a final concentration of 1 μM, and heated to 90 °C for 5 min to denature the RNA. The solution was cooled to room temperature for 20 min, and $MgCl_2$ followed by **5** or **6** was added to obtain a final concentration of 5 mM $MgCl_2$ and 30–50 mM of **5**/**6**. The reaction mixture was incubated at 25 °C. After 0–6 h, the reaction was quenched by precipitating RNA with ethanol. The pellet was redissolved in water, and the unreacted biotinylated cysteine was removed by filtering the solution through a G50 column. The isolated RNA was precipitated with ethanol dissolved in streptavidin agarose coupling buffer (25 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 6.9). The solution was incubated for 45 min with appropriate amounts of streptavidin-derivatized agarose. The mixture containing agarose and RNA was filtered, and the agarose was washed with 4 column volumes of denaturating buffer (8 M urea, 5 mM EDTA, adjusted with K-MOPS to pH 7.4) and water. The amount of RNA linked to the matrix was measured by scintillation counting.

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References and Notes

1. Jenne, A.; Famulok, M. *Chem. Biol.* **1998**, *5*, 23.
2. Suga, H.; Lohse, P. A.; Szostak, J. W. *J. Am. Chem. Soc.* **1998**, *120*, 1151.
3. Wilson, C.; Szostak, J. W. *Nature* **1995**, *374*, 777.
4. Wecker, M.; Smith, D.; Gold, L. *RNA* **1996**, *2*, 982.
5. Ekland, E. H.; Szostak, J. W.; Bartel, D. P. *Science* **1995**, *269*, 364.
6. Zhang, B. L.; Cech, T. R. *Nature* **1997**, *390*, 96.
7. Dai, X. C.; Demesmaeker, A.; Joyce, G. F. *Science* **1995**, *267*, 237.
8. Tsang, J.; Joyce, G. F. *Biochemistry* **1994**, *33*, 5966.
9. Tsang, J.; Joyce, G. F. *J. Mol. Biol.* **1996**, *262*, 31.
10. Seelig, B.; Jäschke, A. *Chem. Biol.* **1999**, *6*, 167.
11. Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54.
12. Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924.
13. Sengle, G.; Eisenführ, A.; Arora, P. S.; Nowick, J. S.; Famulok, M. *Chem. Biol.* **2001**, *8*, 459.
14. Sibi, M. P.; Manyem, S. *Tetrahedron* **2000**, *56*, 8033.
15. Shimizu, S.; Shiozaki, S.; Ohshiro, T.; Yamada, H. *Eur. J. Biochem.* **1984**, *141*, 385.
16. Requena, J. R.; Fu, M. X.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. *Biochem. J.* **1997**, *322*, 317.
17. Sugumaran, M.; Semensi, V.; Dali, H.; Nellaiappan, K. *Arch. Insect Biochem. Physiol.* **1991**, *16*, 31.
18. Carreras, C. W.; Santi, D. V. *Annu. Rev. Biochem.* **1995**, *64*, 721.
19. Montfort, W. R.; Perry, K. M.; Fauman, E. B.; Finer-Moore, J. S.; Maley, G. F.; Hardy, L.; Maley, F.; Stroud, R. M. *Biochemistry* **1990**, *29*, 6964.
20. Walsh, C. T. *Enzymatic Reaction Mechanisms*; W.H. Freeman: San Francisco, CA, 1979.
21. Pitulle, C.; Kleineidam, R. G.; Sproat, B.; Krupp, G. *Gene* **1992**, *112*, 101.
22. Logsdon, N.; Lee, C. G.; Harper, J. W. *Anal. Biochem.* **1992**, *205*, 36.
23. Martin, C. T.; Coleman, J. E. *Biochemistry* **1989**, *28*, 2760.
24. Hausch, F.; Jaschke, A. *Bioconjug. Chem.* **1997**, *8*, 885.
25. Rodebaugh, R.; FraserReid, B.; Geysen, H. M. *Tetrahedron Lett.* **1997**, *38*, 7653.
26. Barany, G.; Albericio, F. *J. Am. Chem. Soc.* **1985**, *107*, 4936.
27. Ordoukhanian, P.; Taylor, J. S. *J. Am. Chem. Soc.* **1995**, *117*, 9570.
28. Givens, R. S.; Kueper, L. W. *Chem. Rev.* **1993**, *93*, 55.
29. Greenberg, M. M. *Tetrahedron Lett.* **1993**, *34*, 251.
30. Hausch, F.; Jaschke, A. *Tetrahedron* **2001**, *57*, 1261.
31. Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925.
32. Caruthers, M. H. *Acc. Chem. Res.* **1991**, *24*, 274.
33. Goodchild *J. Bioconjug. Chem.* **1990**, *1*, 165.
34. Scaringe, S. A.; Wincott, F. E.; Caruthers, M. H. J. *Am. Chem. Soc.* **1998**, *120*, 11820.
35. Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316.
36. McLaughlin, L. W.; Piel, N.; Hellmann, T. *Synthesis* **1985**, 322.
37. Berteina, S.; De Mesmaeker, A. *Synlett* **1998**, 1227.
38. Pons, J. F.; Fauchere, J. L.; Lamaty, F.; Molla, A.; Lazaro, R. *Eur. J. Org. Chem.* **1998**, 853.
39. Muller, D.; Zeltser, I.; Bitan, G.; Gilon, C. *J. Org. Chem.* **1997**, *62*, 411.
40. Kneeland, D. M.; Ariga, K.; Lynch, V. M.; Huang, C. Y.; Anslyn, E. V. *J. Am. Chem. Soc.* **1993**, *115*, 10042.
41. Shih, T. L.; Ruiz-Sanchez, J.; Mrozik, H. *Tetrahedron Lett.* **1987**, *28*, 6015.

42. Smith, A. B.; Fukui, M.; Vaccaro, H. A.; Empfield, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 2071.
43. Oppolzer, W.; Kingma, A. *J. Helv. Chim. Acta* **1989**, *72*, 1337.
44. Jew, S.; Kim, H.; Song, S.; Jang, J.; Park, H. *Heterocycles* **1998**, *47*, 153.
45. Baker, R.; Rao, V. B.; Ravenscroft, P. D.; Swain, C. J. *Synthesis* **1983**, 572.
46. Ainsworth, P. J.; Craig, D.; Reader, J. C.; Slawin, A. M. Z.; White, A. J. P.; Williams, D. J. *Tetrahedron* **1995**, *51*, 11601.
47. Tolbert, T. J.; Wong, C. H. *J. Am. Chem. Soc.* **2000**, *122*, 5421.
48. Pearson, D. A.; Blanchette, M.; Baker, M. L.; Guindon, C. A. *Tetrahedron Lett.* **1989**, *30*, 2739.
49. Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783.