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Divergent Oriented Synthesis For the Design of Reagents for Protein Conjugation

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Abstract

Instead of using diversity oriented syntheses (DOS) to obtain compounds with biological activities, we employed the DOS method to efficiently obtain multifunctional single attachment point (MSAP) reagents for the conjugation to proteins. Acid insensitive functional groups (chelators, fluorochromes) were attached to Lys-Cys-NH_2 or Lys-Lys-βAla-Cys-NH_2 peptide scaffolds. After cleavage from solid supports, the modified peptide intermediates were split and further modified by two solution phase, chemoselective reactions employing the single amine and single thiol presented on the intermediates. MSAP-based fluorochrome–chelates were obtained, some possessing a third functional group like a polyethylene glycol (PEG) polymer or "click chemistry" reactive alkynes and azides. The DOS of MSAP reagents permitted the efficient generation of panels of MSAP reagents that can be used to obtain multifunctional proteins with a single modified amino acid (a single attachment point).

Introduction

The utility of multifunctional nanoparticles, imaging agents, and drug delivery systems has been described on numerous occasions.[1–5] However, the use of independent, function conferring reagents yields multifunctional probes that lack a stoichiometric relationship between the various functional groups attached, since the efficiency of reactions between the substrate and each functional reagent is independent and varies from lot to lot. When one considers the design of multifunctional probes using some protein substrates such as annexin V, an additional problem arises. Here, the attachment of multiple functional groups can lead to a loss of bioactivity for proteins like annexin V which are highly sensitive to the modification of amino acids.[6] Many rDNA engineered proteins also have a very limited number of sites for modification; such proteins often feature a single reactive cysteine thiol distal to the active site which is use for the conjugation of functional groups.

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Supporting Information Available. Brief description of the synthesis of CyAL-5.5. This material is available free of charge via the Internet at http://pubs.acs.org.

To overcome these issues, we developed multifunctional, single attachment point reagents (MSAP reagents) depicted in Figure 1.[7,8] Monofunctional groups (F^1, F^2) and a reactive group (RG) were attached to a peptide scaffold, and the MSAP reagent was then reacted with a substrate to create a multifunctional probe in a single step. Here, we demonstrate two advantages of the MSAP reagent approach. Using a divergent oriented synthetic (DOS) strategy, we show that peptide based intermediates in the MSAP syntheses can be split, to efficiently yield panels of MSAP reagents. Functional groups employed have included fluorochromes, chelators, polymers, and copper(I) azide–alkyne cycloaddition (CuAAC, "click chemistry") reactive groups. Click chemistry reactive groups can be used to obtain ^{18}F labeled probes for positron emission tomography (PET) imaging.[9,10] Second, a bifunctional fluorochrome–chelate MSAP was synthesized and attached to annexin V, an often-used protein in apoptosis research and in molecular imaging. Use of the MSAP reagent yielded a multifunctional annexin V, a protein which is easily inactivated by the attachment of multiple functional groups by modification of multiple amines.[6] We show that the single attachment point strategy of the MSAP reagent yielded a multifunctional annexin V that recognized apoptotic cells. The DOS approach can efficiently yield a wide variety of multifunctional reagents for the design of multifunctional proteins.

Experimental Procedures

MSAP Syntheses. Intermediate Diethylenetriamine Pentaacetic Acid (DTPA)-Lys(Fl)-Cys-NH_2

A solution of 6-(fluorescein-5-carboxyamido)hexanoic acid succinimidyl ester (10.0 mg, 17.0 μmol, 0.7 equiv) in 100 μL anhydrous DMSO was added to a solution of DTPA-Lys-Cys-NH_2 (15.3 mg, 24.6 μmol) in 250 μL anhydrous DMSO containing *N*,*N*-diisopropylethylamine (DIPEA; 12.9 μL, 74.0 μmol, 3 equiv). The reaction mixture was stirred overnight at room temperature and purified by RP-HPLC. The fraction collected was lyophilized. DTPA-Lys(Fl)-Cys-NH_2 (10.5 mg, 9.6 μmol, 56% yield) was obtained as a yellow powder. Mass spectrum: $C_{50}H_{62}N_8O_{18}S$. MW: 1095.1 g · mol^{-1}. Calc exact mass 1094.4; found *m*/*z*: $[M + H]^+$ = 1095.7, $[M + 2H]^{2+}$) 548.6.

DTPA-Lys(Fl)-Cys(NHS^a) (1)

A solution of *N*-[γ-maleimidobutyryloxy] succinimidyl ester (GMBS, 9.5 mg, 33.9 μmol, 6.6 equiv) in 400 μL anhydrous DMF was added to a solution of DTPA-Lys(Fl)-Cys-NH_2 (5.6 mg, 5.1 μmol) in 250 μL anhydrous DMF containing DIPEA (3 μL, 17.2 μmol, 3.4 equiv). The reaction mixture was stirred overnight at room temperature and purified by RP-HPLC. The fraction collected was lyophilized. DTPA-Lys(Fl)-Cys(NHS^a) (4.4 mg, 3.2 μmol, 63% yield) was obtained as a yellow powder. Mass spectrum: $C_{62}H_{74}N_{10}O_{24}S$. MW: 1375.4 g · mol^{-1}. Calc exact mass 1374.5; found *m*/*z*: $[M + H]^+$ = 1375.9, $[M + 2H]^{2+}$ = 688.8.

DTPA-Lys(Fl)-Cys(MAL^a) (2)

A solution of 1,4-bis-(maleimido)butane (BMB, 3.5 mg, 14 μmol, 3 equiv) in 700 μL anhydrous DMSO was added to a solution of DTPA-Lys(Fl)-Cys-NH_2 (5.1 mg, 4.6 μmol) in 230 μL anhydrous DMSO containing DIPEA (1 μL, 5.7 μmol, 1.2 equiv). The reaction mixture was stirred overnight at room temperature and purified by RP-HPLC. The fraction was lyophilized. DTPA-Lys(Fl)-Cys(MAL^a) (3.4 mg, 2.5 μmol, 55% yield) was obtained as a yellow powder. Mass spectrum: $C_{62}H_{74}N_{10}O_{22}S$. MW: 1343.4 g · mol^{-1}. Calc exact mass 1342.5; found *m*/*z*: $[M + H]^+$ = 1344.2, $[M + 2H]^{2+}$ = 672.8.

Intermediate DTPA-Lys(CYAL-5.5)-Cys-NH_2

A solution of CYAL-5.5 succinimidyl ester (20 mg, 25.9 μmol, 0.7 equiv) in 200 μL anhydrous DMF was added to a solution of DTPA-Lys-Cys-NH_2 (23.7 mg, 38 μmol) in 400 μL anhydrous DMF containing DIPEA (39.7 μL, 227.9 μmol, 6 equiv). CYAL-5.5 is a newly synthesized fluorochrome[11] whose optical properties are similar to Cy5.5 and whose structure is shown in Figure 3. A brief description of the synthesis is provided in the Supporting Information. The reaction mixture was stirred for 3 h at room temperature and purified by RP-HPLC. The fraction collected was lyophilized. DTPA-Lys(CYAL-5.5)-Cys(H)-NH_2 (10.6 mg, 7.7 μmol, 30% yield) was obtained as a blue powder. Mass spectrum: $C_{65}H_{85}N_9O_{18}S_3$. MW: 1376.6 g · mol^{-1}. Calc exact mass 1375.5; found *m*/*z*: $[M + H]^+ = 1378.1$, $[M + 2H]^{2+} = 689.2$.

DTPA-Lys(CYAL-5.5)-Cys(NHS^a) (3)

A solution of GMBS (3.7 mg, 13.2 μmol, 2.5 equiv) in 200 μL anhydrous DMSO was added to a solution of (DTPA)-Lys(CYAL-5.5)-Cys(H)-NH_2 (7 mg, 5.2 μmol) in 600 μL anhydrous DMSO containing DIPEA (4 μL, 23.0 μmol, 4.4 equiv). The reaction mixture was stirred for 5 h at room temperature and purified by RP-HPLC. The fraction collected was lyophilized. DTPA-Lys(CYAL-5.5)-Cys(NHS^a) (5.1 mg, 3.08 μmol, 59% yield) was obtained as a blue powder. Mass spectrum: $C_{77}H_{97}N_{11}O_{24}S_3$. MW: 1656.9 g · mol^{-1}. Calc exact mass 1655.6; found *m*/*z*: $[M + H]^+ = 1657.7$ $[M + 2H]^+ = 829.2$.

DTPA-Lys(CYAL-5.5)-Cys(MAL^a) (4)

A solution of BMB (7.8 mg, 31.4 μmol, 12 equiv) in 400 μL anhydrous DMSO was added to a solution of DTPA-Lys(CYAL-5.5)-Cys(H)-NH_2 (3.5 mg, 2.6 μmol) in 300 μL anhydrous DMSO containing DIPEA (2 μL, 11.5 μmol, 4.4 equiv). The reaction mixture was stirred for 6 h at room temperature and purified by RP-HPLC. The fraction collected was lyophilized. DTPA-Lys(CYAL-5.5)-Cys(MAL^a) (3.1 mg, 1.9 μmol, 73% yield) was obtained as a blue powder. Mass spectrum: $C_{77}H_{97}N_{11}O_{21}S_3$. MW: 1624.9 g · mol^{-1}. Calc exact mass 1623.6; found *m*/*z*: $[M + H]^+ = 1626.1$, $[M + 2H]^{2+} = 813.3$.

Intermediate 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid (DOTA)-Lys-Cys-NH_2

DOTA-Lys-Cys-NH_2 was prepared as described.[7] Mass spectrum: $C_{25}H_{46}N_8O_9S$. MW: 634.8 g · mol^{-1}. Calc exact mass: 634.3; found *m*/*z*: $[M + H]^+ = 635.6$.

Intermediate DOTA-Lys(NBD)-Cys-NH_2

Succinimidyl 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate (25 mg, 63.9 μmol, 1.1 equiv) in 500 μL anhydrous DMSO was added to a solution of DOTA-Lys(H)-Cys(H)-NH_2 (35.7 mg, 56.2 μmol) in 500 μL anhydrous DMSO. DIPEA (18 μL, 103.3 μmol, 1.8 equiv) was added and the reaction mixture was stirred for 12 h at room temperature. After semipreparative RP-HPLC purification, the fraction collected was lyophilized. DOTA-Lys(NBD)-Cys(H)-NH_2 (13.6 mg, 14.9 μmol, 26.6% yield) was obtained as an orange powder. Mass spectrum: $C_{37}H_{58}N_{12}O_{13}S$. MW: 911.0 g · mol^{-1}. Calc exact mass 910.4; found *m*/*z*: $[M + H]^+ = 911.6$.

DOTA-Lys(NBD)-Cys(NHS^a) (5)

A solution of GMBS (10.5 mg, 37.5 μmol, 2.5 equiv) in 375 μL anhydrous DMSO was added to a solution of DOTA-Lys(NBD)-Cys(H)-NH_2 (13.6 mg, 14.9 μmol) in 200 μL anhydrous DMSO containing DIPEA (3 μL, 17.2 μmol, 1.2 equiv). The reaction mixture was stirred for 12 h at room temperature. After semipreparative RP-HPLC purification, the fraction collected was lyophilized. DOTA-Lys(NBD)-Cys(NHS^a)-NH_2 (12.3 mg, 10.3 μmol, 69.3% yield) was

obtained as an orange powder. Mass spectrum: $C_{49}H_{70}N_{14}O_{19}S$. MW: 1191.2 g · mol^{-1}. Calc. exact mass 1190.5; found *m*/*z*: $[M + H]^+ = 1191.9$.

Intermediate DOTA-Lys(NIR664)-Cys-NH_2

A solution of NIR-664-*N*-succinimidyl ester from Sigma Aldrich (25.0 mg, 34.5 μmol, 0.6 equiv) in 500 μL anhydrous DMSO was added to DOTA-Lys-Cys-NH_2 (33.9 mg, 53.4 μmol). DIPEA (18 μL, 103.3 μmol, 1.9 equiv) was added and the reaction mixture was stirred overnight at room temperature After semipreparative RP-HPLC purification, the fraction collected was lyophilized. DOTA-Lys(NIR664)-Cys-NH_2 (11.7 mg, 9.4 μmol, 27% yield) was obtained as a blue powder. Mass spectrum: $C_{62}H_{86}N_{10}O_{13}S_2$. MW: 1243.5 g · mol^{-1}. Calc exact mass 1242.6; found *m*/*z*: $[M + H]^+ = 1243.9$, $[M + 2H]^{2+}$) 622.7.

DOTA-Lys(NIR664)-Cys(NHS^a) (6)

A solution of GMBS (5.7 mg, 20.4 μmol, 2.2 equiv) in 200 μL anhydrous DMSO was added to a solution of DOTA-Lys(NIR664)-Cys-NH_2 (11.7 mg, 9.4 μmol) in 200 μL anhydrous DMSO containing DIPEA (3 μL, 17.2 μmol, 1.8 equiv). The reaction mixture was stirred overnight at room temperature. After semi-preparative RP-HPLC purification, the fraction collected was lyophilized. DOTA-Lys(NIR664)-Cys(NHS^a)-NH_2 (12.3 mg, 8.1 μmol, 86% yield) was obtained as a blue powder. Mass spectrum: $C_{74}H_{98}N_{12}O_{19}S_2$. MW: 1523.8. Calc exact mass 1522.7; found *m*/*z*: $[M + H]^+ = 1524.7$; $[M + 2H]^{2+} = 762.7$.

DTPA-Lys(FITC)-Lys(Alkyne)-βAla-Cys(NHS^a) (7)

The linear (DTPA)-Lys(FITC)-Lys-βAla-Cys-NH_2 peptide was synthesized as described.[7] A solution of 4-pentynoic acid *N*-succinimidyl ester (48.8 mg; 250 μmol; 10 equiv) in 500 μL anhydrous DMSO was added to 250 μL of a solution of DTPA-Lys(FITC)-Lys-βAla-Cys-NH_2 (31.0 mg; 25.6 μmol) in anhydrous DMSO containing DIPEA (4.5 μL; 25.8 μmol; 1.0 equiv). The reaction mixture was stirred for 48 h at room temperature. The reaction mixture was purified by RP-HPLC, and the fraction collected was lyophilized. DTPA-Lys(FITC)-Lys (Alkyne)-βAla-Cys (11.2 mg, 8.7 μmol, 34% yield) was obtained as a yellow powder. ($C_{58}H_{73}N_{11}O_{19}S_2$) Calc. exact mass 1291.5; found *m*/*z*: $[M + H]^+ = 1292.9$, $[M + 2H]^{2+}$= 647.2. DTPA-Lys(FITC)-Lys(Alkyne)-βAla-Cys (11.2 mg; 8.7 μmol) was added to 264 μL of a solution of *N*-[γ-maleimidobutyryloxy] succinimidyl ester (GMBS; 7.4 mg; 26.4 μmol; 3 equiv). DIPEA (1.5 μL; 8.6 μmol; 1 equiv) was added. The reaction mixture was stirred for 48 h at room temperature. The reaction mixture was purified by RP-HPLC, and the fraction collected was lyophilized. DTPA-Lys(FITC)-Lys(Alkyne)-βAla-Cys(NHS) (9.8 mg, 6.2 μmol, 71% yield) was obtained as a yellow powder. ($C_{70}H_{85}N_{13}O_{25}S_2$) MW: 1572.6. Calc exact mass 1571.5; found *m*/*z*: $[M + 2H]^{2+} = 787.3$.

DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys (8)

A solution of PEG_8-NHS ester (75 μmol; 3.9 equiv) in 300 μL anhydrous DMSO was added to DTPA-Lys(FITC)-Lys(H)-βAla-Cys(H)-NH_2 (23.4 mg; 19.3 μmol). After addition of DIPEA (3.4 μL; 1 equiv), the reaction mixture was stirred for 48 h at room temperature. The reaction mixture was purified by semipreparative RP-HPLC, and the fraction collected was lyophilized. DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys (3.6 mg, 2.24 μmol, 11.6% yield) was obtained as a yellow powder. ($C_{71}H_{103}N_{11}O_{27}S_2$) MW: 1606.8. Calc exact mass: 1605.7; found *m*/*z*: $[M + H]^+ = 1607.8$, $[M + 2H]^{2+} = 804.4$, $[M + 3H]^{3+} = 536.6$.

DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys(NHS^a) (9)

GMBS was added to a solution of DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys(H)-NH_2 (3.6 mg; 2.24 μmol) in 40 μL anhydrous DMSO containing DIPEA. The reaction mixture was stirred for 24 h at room temperature. The reaction mixture was purified by semipreparative RP-HPLC,

and the fraction collected was lyophilized. DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys(NHS^a) was obtained as a yellow powder. ($C_{83}H_{115}N_{13}O_{33}S_2$) MW: 1887.0. Calc exact mass 1885.7; found *m*/*z*: $[M + H]^+ = 1888.1$, $[M + 2H]^{2+} = 944.5$, $[M + 3H]^{3+} = 630.0$.

DTPA-Lys(FITC)-Lys(PEG_8-N_3)-βAla-Cys(MAL^a) (10)

DTPA-Lys(FITC)-Lys(H)-βAla-Cys(H)-NH_2 (27.2 mg; 22.4 μmol) was added to 3.3 mL of a solution of 1,4-bismale-imidobutane (16.3 mg; 39.8 μmol; 3.0 equiv) containing DIPEA (5 μL; 28.7 μmol; 1.3 equiv). The reaction mixture was stirred for 24 h at room temperature. A solution of azido-dPEG$_8$-NHS ester (42.9 mg; 75 μmol; 3.3 equiv) in 300 μL anhydrous DMSO was added. The reaction mixture was stirred for another 46 h at room temperature. The reaction mixture was purified by RP-HPLC, and the fraction collected was lyophilized. DTPA-Lys (FITC)-Lys(PEG_8-Azido)-βAla-Cys(Maleimide) (8.6 mg, 4.5 μmol, 20% yield) was obtained as a yellow powder. ($C_{84}H_{116}N_{16}O_{31}S_2$) MW: 1910.0. Calc exact mass 1908.7; found *m*/*z*: $[M + H]^+ = 1910.3$, $[M + 2H]^{2+} = 955.8$.

MSAP-Annexin V Synthesis

To 100 μL of annexin V (0.354 mg, 9.83 nmoles) in 0.05 M $NaHCO_3$ buffer, pH 8.1, was added 5.5 μL of **3** (55 nmoles) in DMSO. After 15 min at room temperature in the dark, the mixture was applied to a PD-10 column in PBS. The high molecular weight fraction was collected. The number of MSAP's per annexin (0.7) was determined from absorbances at 280 nm (annexin V) and 682 nm (NIR664 fluorochrome). APC-annexin V was from InVitrogen. Cells were stained and analyzed by FACS as described.[12]

Results and Discussion

The divergent strategy developed to obtain a series of bifunctional fluorochrome–chelate MSAP's from a dipeptide scaffold is shown in Figure 2. The Fmoc-Lys(Boc)-Cys(Trt) peptide was prepared manually on a solid support, on a scale sufficient to support the number of individual MSAP's to eventually be synthesized (about 0.2 mmol). Removal of the N-terminal Fmoc allowed a first functional group (F^1) to be attached to the N-terminal end of the dipeptide. In the case of the current fluorochrome–chelate MSAP's, *t*-butyl protected DTPA or DOTA was coupled to the N-terminus, followed by cleavage and storage of the DTPA-Lys-Cys or DOTA-Lys-Cys dipeptide intermediates. The latter featured a single primary amine, which was used for the attachment of a fluorochrome (NBD, Fl, NIR664, or CYAL-5.5) in solution, and a single thiol, which was subsequently modified with a commercially available bifunctional cross-linking agent for the introduction of a reactive group (RG). A total of eight maleimide (MAL) or *N*-hydroxysuccinimide ester (NHS) fluorochrome–chelate MSAP's were obtained from common intermediates by two chemoselective ligations. A summary of the MSAP's prepared with this strategy is provided in Table 1, with the structures of the functional groups and reactive groups, and the abbreviations employed for them shown in Figure 3. The "a" or "b" designation in MAL^a or NHS^a refers to the use of slightly different cross-linkers. The Fmoc-Lys(Boc)-Cys(Trt) peptide used in the current study has also been used to obtain fluorochrome–biotin MSAP reagents for protein biotinylation.[8] As shown with compounds **1** through **4**, either MAL or NHS reactive groups can be used with various combinations of chelate and fluorochrome functional groups. Consequently, for MSAP's bearing DOTA together with NBD or NIR664 functional groups, we provide syntheses for only the NHS ester versions (compounds **5** and **6**). As shown in Table 1, the MSAP design permits reactive groups and functional groups to be attached to the primary amine or to the thiol which remain after the N-terminal amine has been reacted with a functional group on the solid phase. Thus the MAL or NHS reactive groups were attached to the cysteine thiol (to yield compounds **1**–**6**) or to the epsilon amine of lysine (to yield Biotin-Lys(NHS^b)-Cys(AcmFl) and Biotin-Lys (MAL^b)-Cys(AcmFl)).

An advantage of the MSAP strategy is its flexibility, allowing variable numbers and types of functional groups to be employed in different combinations. To increase the number of functional groups from 2 to 3, we employed the tetrapeptide Lys-Lys-βAla-Cys-NH_2 scaffold; see Figure 4. After the solid phase synthesis of Fmoc-Lys(ivDde)-Lys-(Boc)-βAla-Cys(Trt), *t*-butyl protected DTPA and fluorescein isothiocyanate were sequentially coupled to the deprotected N-terminal and epsilon amino group of the N-terminal lysine, respectively. Cleavage and deprotection yielded a fluorochrome–chelate tetrapeptide intermediate that again featured a single primary amine and a single thiol, enabling two additional chemoselective reactions to be performed in solution. Here the thiol group was used for the attachment of a reactive group (RG), and the remaining amino group was used to attach PEG_8 (400 Da) or PEG_{5000} (5000 Da), to vary the size and hydrophilicity of the MSAP reagent and of the resulting imaging probe. In addition, we employed as functional groups azide and alkyne groups suitable for copper(I) azide–alkyne cycloaddition (CuAAC, click chemistry). Thus functional groups can report on probe position (chelates for radioactive metals, fluorochromes), alter probe the size and hydrophilicity (various PEG's), or enable further chemical reactions. The panel of trifunctional MSAP's synthesized is summarized in Table 2.

To illustrate the ability of a fluorochrome–chelate MSAP to generate a bioactive, multifunctional protein, we reacted the DTPA-Lys(CYAL-5.5)-Cys(NHS^a) MSAP (**3**) with an annexin V substrate. We chose annexin V because of a long-term interest in developing a clinically useful multifunctional annexin V, one with a high affinity metal chelator optimized for clinical use, but which could also be used in fluorescence based research applications. In the clinic, radioactive annexin V's have been used to image apoptosis in a wide range of conditions,[13,14] while fluorescent annexin V's have often been used to image apoptosis in animal models[15] and for apoptosis research.[16] Annexin V is a 33 kDa protein which undergoes inactivation with the modification of more than one amino group per mole of protein.[6] We therefore modified annexin V with 0.7 mols of MSAP per mole of protein as shown in Figure 5. The single attachment point feature of the MSAP permitted the attachment of 1.4 mols of functional groups per mole of annexin V (0.7 DTPA plus 0.7 CYAL-5.5) with the modification of only 0.7 amine per mole (Figure 5A). The MSAP–annexin V conjugate bound camptothecin-treated A549 and Jurkat T cells (Figure 5B and C, respectively). The binding was blocked by unlabeled annexin V, indicating the bioactivity of the MSAP–annexin V.

The peptide scaffold approach used in the design of MSAP reagents permits highly variable numbers and combinations of functional groups to be employed, albeit with some restrictions we should note. First, the functional groups attached to the peptide scaffold during the solid phase steps must survive the conditions for solid phase peptide synthesis and those for deprotection and cleavage from the resin. Second, the solid phase synthesis method requires the use of an excess of reagent and can be less efficient than reactions performed in solution. This becomes an important issue when the use of costly functional groups is considered.

The use of trifunctional MSAP's with clickable alkynes and azides as functional groups permits the attachment of MSAP reagents to substrates, while offering a group for further covalent chemistry. The reaction of the MSAP with an epsilon amine of a lysine on a protein substrate, for example, provides a probe whose detection can be obtained through the chelator (radioactive metal) and fluorochrome (fluorescence). Yet after consuming the amine, a "clickable" functional group is available for further modification.

A significant benefit of fluorochrome–chelate MSAP's was the use of its fluorochrome absorbance to monitor disposition of the chelate. We employed the absorbance of CYAL-5.5 to determine the number of DTPA chelates attached per mole of annexin V. For low numbers of chelates per mole of protein, fluorescence can also be employed as we have shown with a biotin–fluorochrome MSAP reagent.[8]

MSAP reagents are useful for the design of multifunctional probes using small protein substrates (5–50 kDa), where there are a small of number of amino acid residues are available for modification with the retention of bioactivity. To illustrate the ability of MSAP's to address this problem, a fluorochrome–chelate MSAP reagent was reacted with the amines of annexin V in a random fashion, to obtain an MSAP–annexin V with 1.4 mols of functional groups and 0.7 mols of modified lysine side chains per mole of protein. A similar limited number of reactive sites occurs with many rDNA-engineered proteins, which are designed for a site-specific modification at a single reactive thiol placed distal to the active site. Here only a single attachment point, a cysteine thiol, is available for the conjugation of all the functional groups needed to obtain a multifunctional protein.[17] In these cases, multifunctional MSAP reagents with maleimide reactive groups can yield small, multifunctional rDNA proteins in a single step.

In conclusion, we have demonstrated a DOS strategy to obtain panels of multifunctional single attachment point (MSAP) reagents. The strategy consisted of a solid phase synthesis of modified peptide intermediates, cleavage from the solid phase, and solution phase reactions with single amines and single thiols present on these intermediates. This DOS strategy allowed chemistry of functional groups chosen to vary widely and allowed them to be employed in different combinations. Functional groups included fluorochromes, chelates, polymers, and click reactive groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMB	1,4-bis(maleimido)butane
CPT	camptothecin
DIPEA	*N*,*N*-diisopropylethylamine
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DSS	disuccinimidyl suberate
DTPA	diethylenetriamine pentaacetic acid
FITC	fluorescein isothiocyanate
GMBS	*N*-[γ-maleimidobutyryloxy] succinimidyl ester
MAL	maleimide
MSAP	multifunctional single attachement point
NHS	*N*-hydroxysuccinimide
PEG	polyethylene glycol
RG	reactive group
RGD	Arg-Gly-Asp
RP-HPLC	reverse phase high performance liquid chromatography

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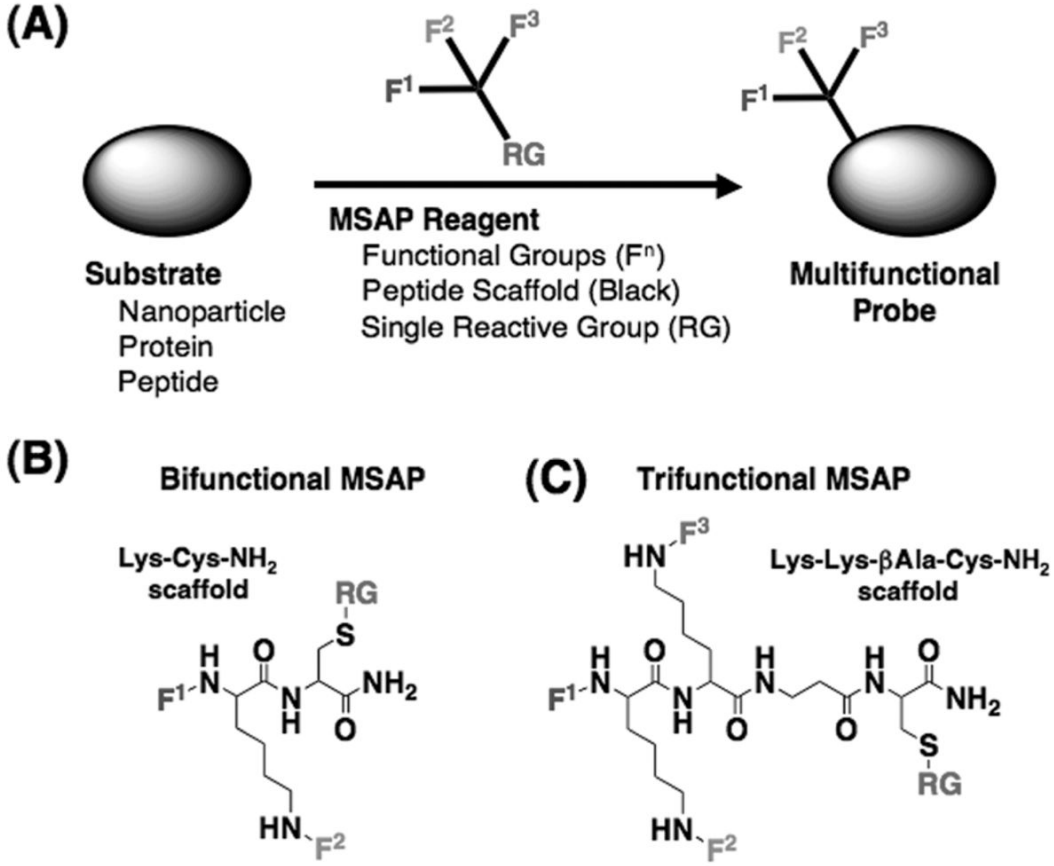


Figure 1.

Multifunctional single attachment point (MSAP) reagents, concept and general structures. (A) Schematic view of the MSAP concept. An MSAP reagent, composed of three functional groups (F^1, F^2, and F^3) and a reactive group (RG), is reacted with a substrate to yield a multifunctional probe in one reaction step. The substrate can be from different classes of bioactive materials (nanoparticle, polymer, protein, peptide). (B) Bifunctional MSAP reagents consist of two functional groups (F^1, F^2) and a reactive group (RG) attached to a dipeptide Lys-Cys-NH_2 scaffold. Trifunctional MSAP reagents consist of three functional groups (F^1, F^2, and F^3) and a reactive group attached to a tetrapeptide Lys-Lys-βAla-Cys-NH_2 scaffold.

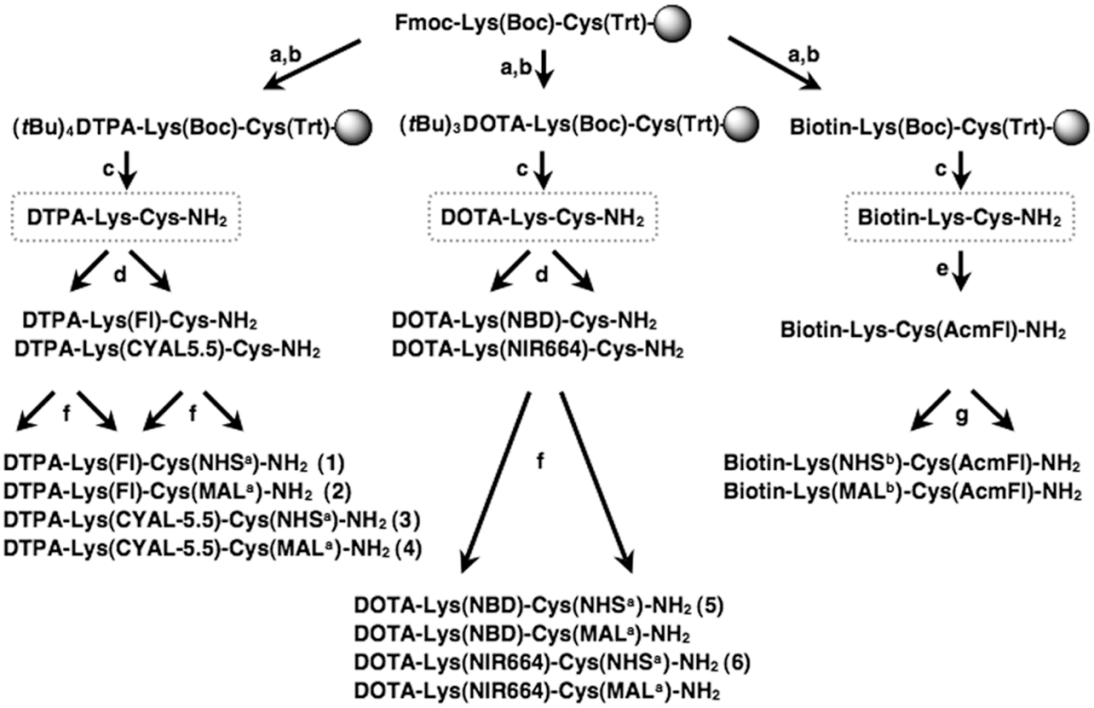


Figure 2.

Divergent synthetic strategy for synthesizing fluorochrome–chelate MSAP's. The chelators (DOTA or DTPA, the F^1 group in Figure 1B) were attached on the solid phase. After deprotection and cleavage, the F^1-Lys-Cys-NH_2 peptides (gray highlights) served as common intermediates to prepare a total of eight fluorochrome–chelator MSAP's. Fluorochromes (F^2, Figure 1B) were 6-(fluorescein-5-carboxyamido)hexanoyl (Fl), CYAL-5.5, NBD, or NIR664. The reactive groups (RG, Figure 1B) were an NHS ester (NHS) or a maleimide (MAL). Two additional fluorochrome–biotin MSAP's using the same peptide scaffold are also shown and are from ref [8]. Reaction conditions Fmoc/*t*Bu SPPS: (a) Piperidine/DMF; (b) (*t*Bu-Protected) F^1, PyBOP, DIPEA, DMF; (c) TFA/TIS/H_2O/EDT; (d) F^2-NHS, DIPEA, DMSO/DMF; (e) fluorescein-5-iodoacetamide, DIPEA, DMF; (f) cross-linking agent (GMBS or BMB), DIPEA, DMSO/DMF; (g) cross-linking agent (DSS or GMBS), DIPEA, DMSO/DMF.

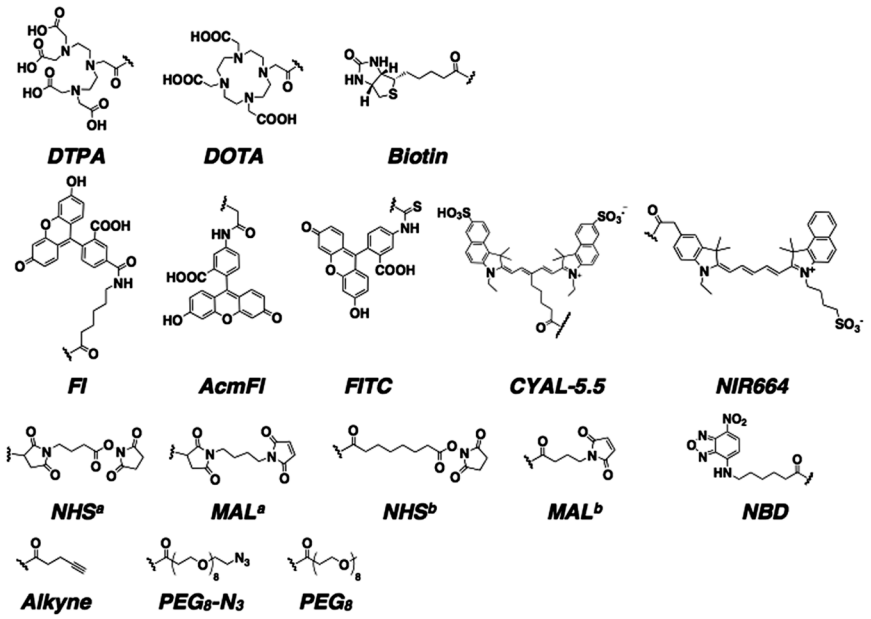


Figure 3.
Structure of functional groups used in MSAP reagents.

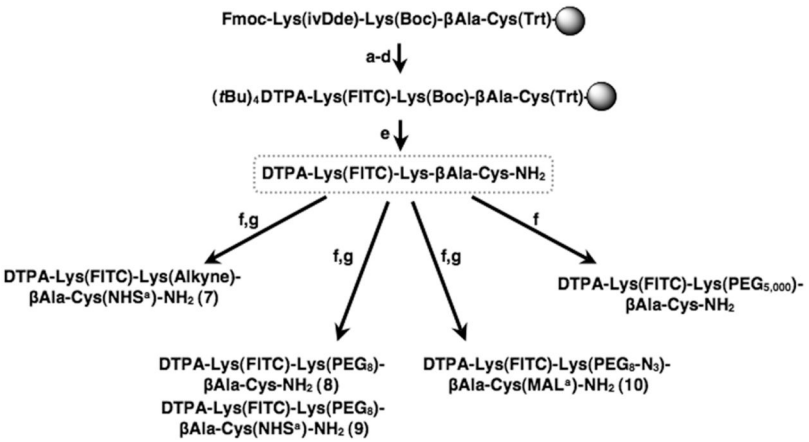


Figure 4.

Divergent synthetic strategy for synthesizing trifunctional MSAP's. After deprotection and cleavage, the DTPA-Lys(FITC)-Lys-βAla-Cys-NH_2 peptide (a common intermediate, gray highlight) was used to prepare five MSAP's. Here F^1 = DTPA, F^2 = FITC; see Figure 1B. Different PEG's (F^3; Figure 1B) and different reactive groups were attached. MSAP functional groups can provide multimodal detection (DTPA, FITC), modify substrate properties ($PEG_{5000,}$ PEG_8), or equip a probe with groups for subsequent reactions using click chemistry. Reaction conditions Fmoc/tBu SPPS: (a) piperidine/DMF; (b) (tBu)$_4$DTPA, PyBOP, DIPEA, DMF; (c) hydrazine/DMF; (d) FITC, DIPEA, DMF; (e) TFA/TIS/H_2O/EDT; (f) F^3-NHS, DIPEA, DMSO/DMF; (g) cross-linking agent (GMBS or BMB), DIPEA, DMSO/DMF.

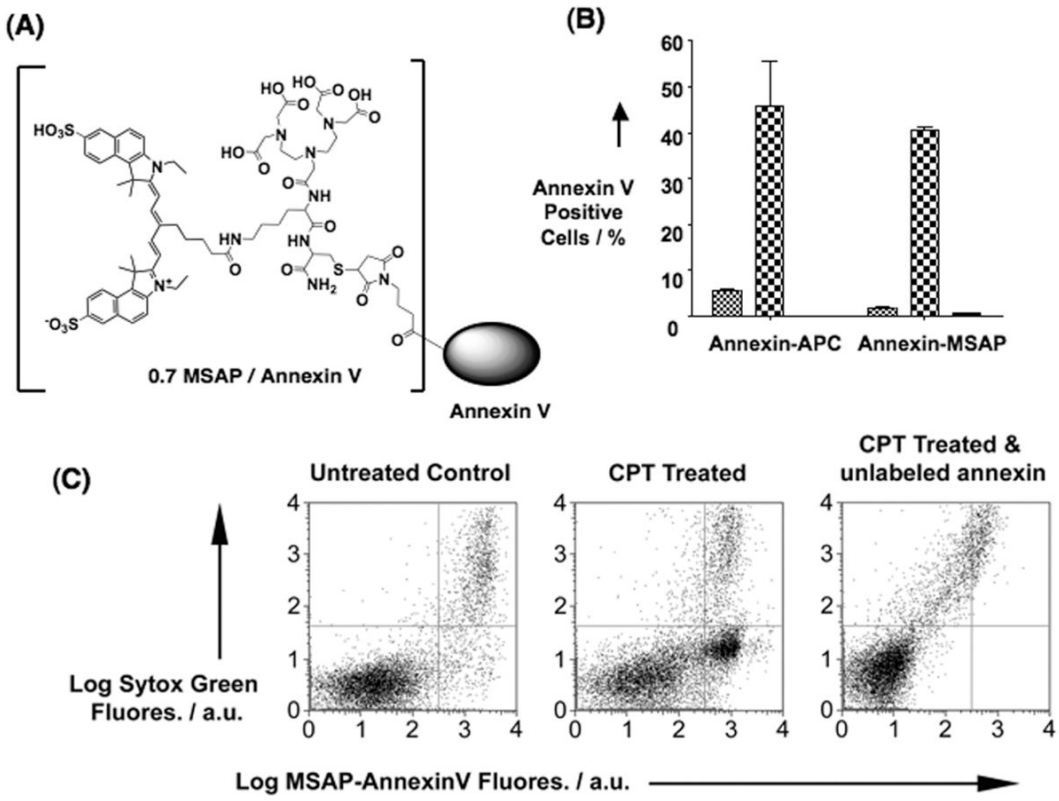
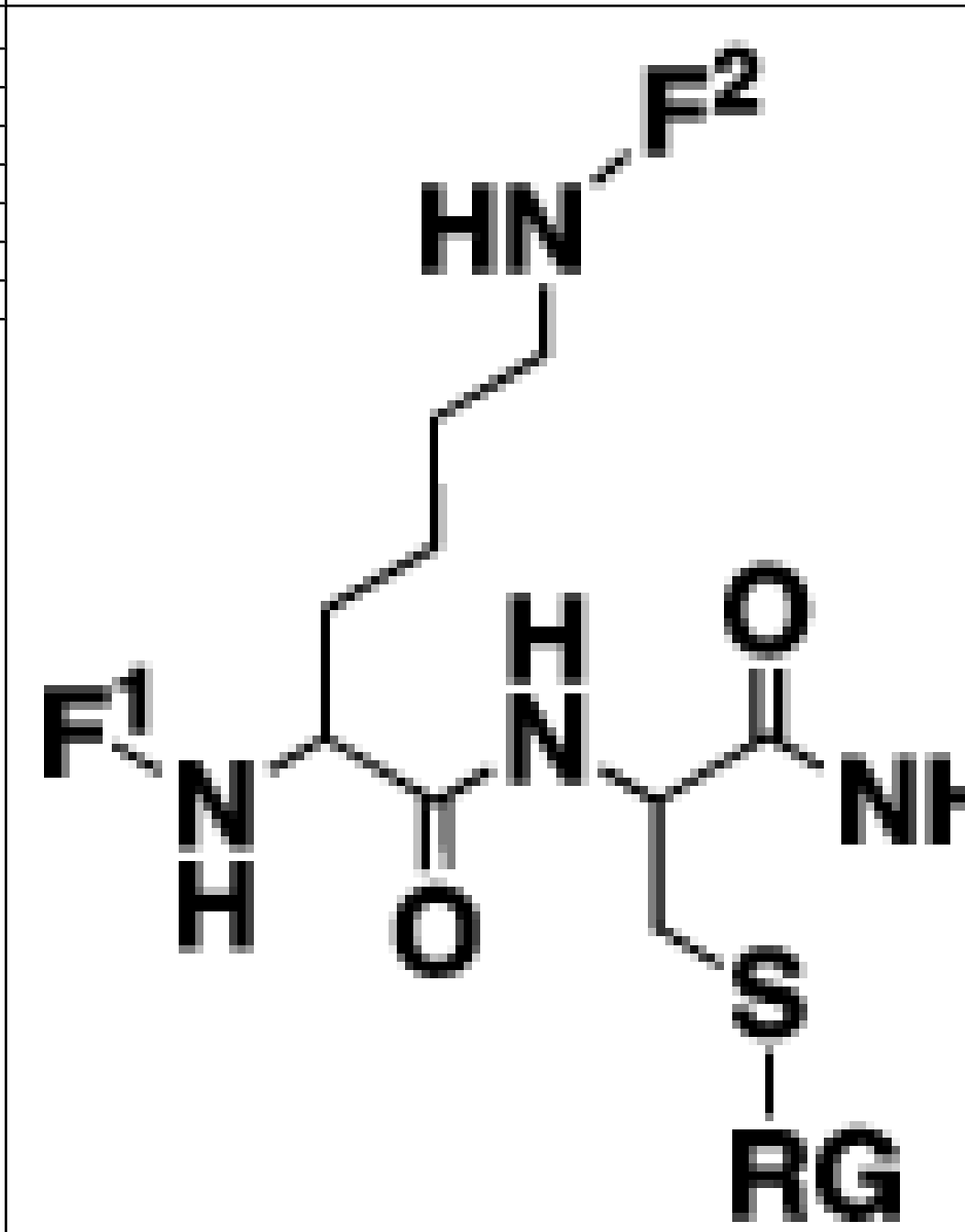


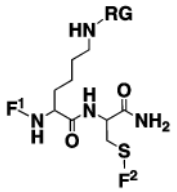
Figure 5.

Detection of apoptotic and necrotic cells using a multifunctional MSAP–annexin V by dual wavelength FACS. (A) Schematic depiction of the MSAP–annexin V probe. Compound (**3**) was reacted with annexin V to obtain an annexin V modified with 0.7 MSAP per mole of protein (0.7 fluorochrome, 0.7 DTPA). (B) MSAP–annexin V bound to apoptotic or necrotic A549 cells. (C) MSAP–annexin V bound to apoptotic Jurkat T cells. Apoptosis (right, bottom quadrant) was induced by 5 μM camptothecin (CPT) in parts B and C.

Table 1

Summary of Bifunctional MSAP's Based On the Lys-Cys-NH_2 Peptide Scaffold

Designation	Scaffold
DTPA-Lys(NBD)-Cys(NHS[a])*	
DTPA-Lys(F1)-Cys(NHS[a]), Compound (**1**)	
DTPA-Lys(F1)-Cys (MAL[a]) (**2**)	
DTPA-Lys(CYAL-5.5)-Cys(NHS[a]) (**3**)	
DTPA-Lys(CYAL-5.5)-Cys(MAL[a]) (**4**)	
DOTA-Lys(NBD)-Cys(NHS[a]) (**5**)	
DOTA-Lys(NBD)-Cys(MAL[a])	
DOTA-Lys(NIR664)-Cys(NHS[a]) (**6**)	
DOTA-Lys(NIR664)-Cys(MAL[a])	

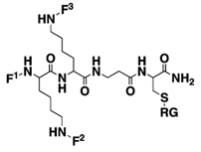
Designation	Scaffold
Biotin-Lys(NHS[b])-Cys(AcmF1)**	
Biotin-Lys(MAL[b])-Cys(AcmF1)**	

*Reference 7.

**Reference 8.

Table 2

Summary of Trifunctional MSAP's Based on the Lys-Lys-βAla-Cys-NH_2 Peptide Scaffold

Designation	Scaffold	F^1	F^2	F^3	Reactive Group
DTPA-Lys(FITC)-Lys($PEG_{5,000}$)-βAla-Cys,[7]		DTPA	FITC	PEG_{500}	Thiol
DTPA-Lys(FITC)-Lys(Alkyne)-βAla-Cys(NHS[a]), Compound (**7**)		DTPA	FITC	Alkyne	NHS[a]
DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys (**8**)		DTPA	FITC	PEG_8	Thiol
DTPA-Lys(FITC)-Lys(PEG_8)-βAla-Cys(NHS[a]) (**9**)		DTPA	FITC	PEG_8	NHS[a]
DTPA-Lys(FITC)-Lys(PEG_8-N_3)-βAla-Cys(MAL[a]) (**10**)		DTPA	FITC	PEG_8-Azido	MAL[a]