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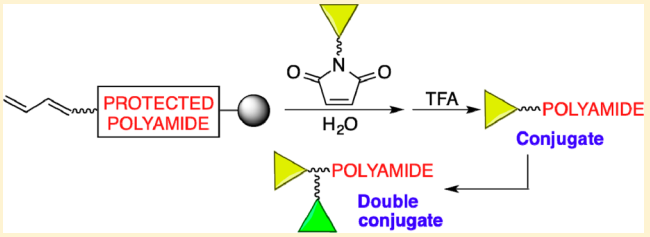
On-Resin Conjugation of Diene–Polyamides and Maleimides via Diels–Alder Cycloaddition

Omar Brun, Xavier Elduque, Enrique Pedroso, and Anna Grandas*

Departament de Química Orgànica, Facultat de Química and IBUB, Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

Supporting Information

ABSTRACT: The reaction between maleimides and resin-linked diene–polyamides allows the latter to be used in the preparation of conjugates. Conjugation takes place by reacting the insoluble, hydrophobic diene component either with water-soluble dienophiles or with dienophiles requiring mixtures of water and organic solvents. Experimental conditions can be adjusted to furnish the target conjugate in good yield with no need of adding large excesses of soluble reagent. In case protected maleimides are used, maleimide deprotection and Diels–Alder cycloaddition can be simultaneously carried out to render conjugates with different linking positions. On-resin conjugation is followed by an acidic treatment that removes the polyamide protecting groups with no harm to the cycloadduct, in contrast with the unreacted diene that is indeed degraded under these conditions. Cycloadducts incorporating suitable functional groups can undergo subsequent additional conjugation reactions in solution to furnish double conjugates.



INTRODUCTION

Chemical reactions allowing natural or synthetic biomolecules to be appended with reporter groups (such as fluorescent tags, spin labels, or biotin), radical-generating reagents, metal complexes, cell-penetrating entities, or specific recognition motifs have been the focus of extensive research over the past few decades.[1] In the resulting new entities, commonly referred to as conjugates, biomolecules are expected to be endowed with new or improved properties. In order to prevent the results of conjugate-involving experiments from being misinterpreted, as well as premature loss of conjugation synergies, conjugates should have a defined structure and be stable.

One of the oldest and most extensively used conjugation reactions is the Michael-type reaction between a thiol and a maleimide.[2] It takes place quickly and in high yield, is water compatible, does not require any catalyst, and does not generate byproducts. Therefore, it fulfills the basic requirements of click reactions.[3] Yet, the thiol–maleimide reaction generates two diastereomers, and different recent publications have revealed that the resulting succinimide may undergo thiol exchange and hydrolysis.[4−7] The Cu(I)-catalyzed Huisgen cycloaddition between an azide and an alkyne has become the par excellence click reaction after it was described that Cu(I) accelerates the cycloaddition and prevents formation of the 1,5-regioisomer.[8,9] The addition of Cu(I)-stabilizing entities accelerates the reaction and prevents formation of oxidizing species[10] and has been key in the preparation of all types of conjugates linked through stable 1,2,3-triazoles. However, complete elimination of the metal catalyst may be difficult to achieve,[11,12] and optimization of reaction conditions may be highly demanding.[13,14] Suitably modified strained alkynes (such as cyclooctynes) react with azides in the absence of Cu(I),[15,16] in other words in biocompatible conditions, but the reaction gives a mixture of regioisomers.

The Diels–Alder (DA) cycloaddition has been successfully used to prepare bioconjugates. It offers the advantage of being totally chemoselective and byproduct free and can be conducted in (and be accelerated by) water, and hence, it can also be classified as a click reaction. The DA cycloaddition is known to furnish a mixture of isomers in which those with the endo configuration usually predominate.[17] With respect to the Michael-type and Cu(I)-catalyzed Huisgen cycloaddition, it offers the advantage of providing stable conjugates that do not undergo exchange with reagents present in biological media and that of not requiring metal catalysts if performed in aqueous media, respectively.[18−20] Moreover, cycloadducts formed in DA reactions are smaller than those formed in copper-free azide–cyclooctyne reactions, especially in the case of cyclooctynes activated by mono- or dibenzo substitution.

Bioconjugates have been obtained in solution from DA reactions, affording both carbon–carbon[21−29] and carbon–heteroatom bonds[30−32] as well as using the inverse electron demand version.[33−36] The DA reaction is in principle reversible,[37] but most cycloadducts are sufficiently stable to withstand chemical manipulation and use in physiological media because the retro-DA reaction typically requires fairly harsh conditions.[37]

In principle, any of the two reacting groups (diene or dienophile) can be linked to either of the conjugate

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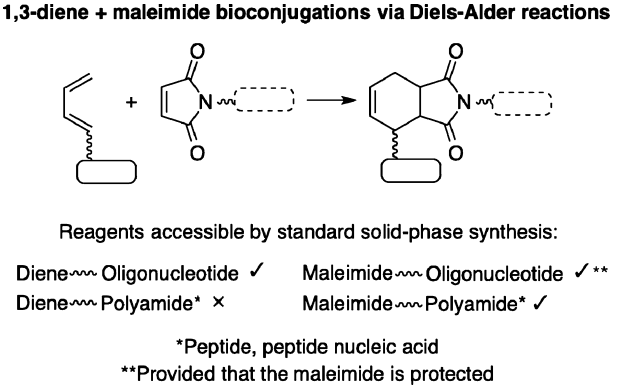
components. In the case of molecules routinely prepared by solid-phase synthesis, such as peptides and oligonucleotides, the most straightforward alternative is to introduce the additional group required for conjugation while they are still linked to the solid matrix. Yet, the compatibility of dienes and dienophiles with the reaction conditions that deprotect the oligomer and cleave the oligomer–resin bond has to be considered. For instance, maleimides (which are the dienophiles most commonly used in bioconjugations) are stable to the acidic treatment that deprotects peptides but not to the reaction with ammonia that deprotects oligonucleotides. As a result, maleimido–oligonucleotides can only be on-resin assembled provided that the maleimide moiety is protected.[38] Dienes are not degraded by the ammonia treatment, and dienes conjugated with electron-withdrawing groups remain stable to polyamide acidic deprotection conditions.[39,40] However, alkyl-substituted 1,3-dienes, which react more quickly with dienophiles in prototype DA cycloadditions, are in principle degraded by strong acids (Scheme 1).

Scheme 1. Bioconjugation Reactions Involving Alkyl-Substituted 1,3-Dienes and Maleimides[a]



[a]General structures of the reagents and cycloadduct, and summary of the permitted derivatizations.

There are no precedents in the literature reporting the synthesis and use of diene–PNAs (peptide nucleic acids) in DA cycloadditions, and no general alternative for the solid-phase synthesis of alkyl-substituted 1,3-diene–peptides (or any diene–polyamide whose final deprotection reaction requires strong acids) has so far been described. The few reports involving diene–peptides have some limitations. The Madder group could obtain furan-derivatized peptides by solid-phase synthesis provided that an aromatic residue was placed next to the furan-containing building block;[41] otherwise, furan was reduced by the triisopropylsilane (TIS) scavenger typically present in the final peptide deprotection mixture. This group also reacted maleimides with resin-linked furans, but the resulting cycloadducts partially underwent reversion to the starting materials.[42] The Waldmann group prepared 1,3-diene–peptides by solid-phase assembly on a sulfamylbutyryl linker resin using Fmoc–amino acids (Fmoc = 9-fluorenylmethoxycarbonyl).[26] However, their methodology is only compatible with glycine at the *C*-terminus and Fmoc–amino acids with side chain protecting groups removable under very mild acidic conditions, which excludes the presence of some trifunctional amino acids because the required derivatives are not commercially available.

Therefore, the DA cycloaddition between dienophiles and diene–polyamides is the only combination in which one of the reagents is not easily available, since the instability of 1,3-diene–polyamides to acids precludes their solid-phase synthesis using standard procedures.

With the aim of extending the DA-based conjugation toolbox, we decided to examine the possibility of attaching alkyl-substituted 1,3-dienes to resin-linked peptides and PNAs and carrying out the DA cycloaddition on the solid matrix. This alternative is simple from the operational point of view, of general application, and offers the advantage of preventing dienes from being exposed to acid-containing deprotection mixtures and diene–polyamides from having to be purified before the conjugation step. The final deprotection treatment takes place after formation of the cycloadduct, which remains stable under these conditions. Moreover, cycloadducts derived from the reaction between 1,3-dienes and maleimides are more stable than those formed by reacting maleimides and furans.[26,42,43]

However, the fact that the dienophile-containing moiety might possibly be a water-soluble biomolecule raised some doubts as to the feasibility of this synthetic alternative. To our knowledge, on-resin DA reactions[44] have always been carried out in organic solvents, even on hydrophilic resins.[45] The use of water is indeed advantageous, because the reaction takes place more quickly and in milder conditions than in organic solvents,[18−20] but the outcome of cycloadditions between water-soluble maleimide-containing compounds and hydrophobic diene-[protected polyamide]-resins performed in water (or solvents with a high water content) was not obvious.

Here we describe that such on-resin DA reactions in water (Scheme 2a) do provide the target conjugates, allowing diene–polyamides to be used in DA reactions. We also report that the scope of conjugation alternatives can be broadened by using protected maleimide derivatives and simultaneously carrying out maleimide deprotection and conjugation (Scheme 2b). This approach has been used to obtain conjugates with different linking sites and in reactions involving maleimides derivatized with tagging agents such as fluorophores and biotin, which are sparingly soluble in water. In case conjugates assembled by on-resin DA cycloaddition incorporate appropriate functional groups, an additional chemoselective conjugation reaction can be carried out to yield doubly derivatized molecules.

RESULTS AND DISCUSSION

Preliminary Experiments, Choice of the Solid Matrix, and Analysis of the Results. It is well known that conjugated polyenes react with acids and undergo addition reactions. As stated above, dienes conjugated with electron-withdrawing groups such as sorbic acid can be submitted to peptide deprotection conditions with no harm to the conjugated system,[39,40] but alkyl-substituted 1,3-dienes cannot be presumed to exhibit the same behavior. To definitely assess whether alkyl-substituted 1,3-dienes would or not withstand typical peptide deprotection conditions, $H_2C{=}CH{-}CH{=}CH{-}CH_2{-}CH_2{-}CO$-Ala-Tyr(*t*Bu)-Lys(Boc)-Rink amide resin (Boc = *tert*-butoxycarbonyl) was treated with a 95:2.5:2.5 TFA/TIS/water mixture for 2 h and the resulting crude analyzed by HPLC-MS. The crude was very complex, and the main peaks were peptides linked to products resulting from hydration and reduction of the 1,3-diene, as well as peptides containing only fragments of the diene linker chain (see Supporting Information, section 3).

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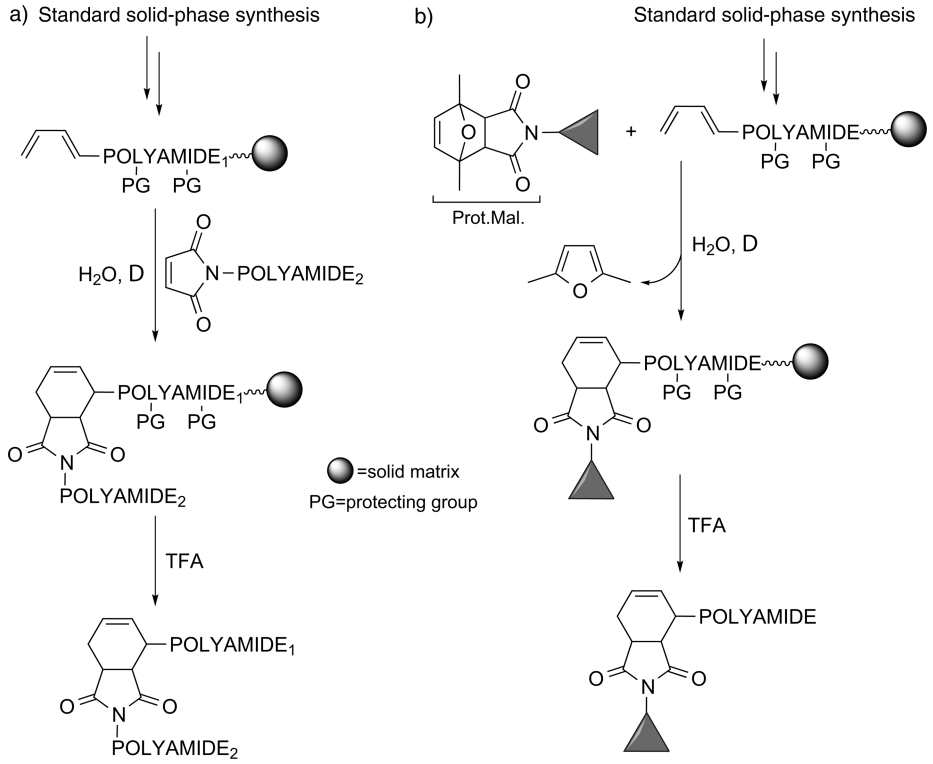
Scheme 2. General Schemes for (a) Solid-Phase DA Cycloaddition between Maleimides and Diene–Polyamides and (b) the on-Resin Conjugation between Protected Maleimides and Diene–Polyamides[a]



POLYAMIDE$_1$	POLYAMIDE$_2$
PNA	Peptide
Peptide	PNA

POLYAMIDE	▲
PNA	Peptide*, biotin, fluorophore
Peptide	Fluorophore, biotin

[a]In both cases, the table below each of the schemes shows the moieties combined in the different conjugates. *Linear or cyclic peptides, with the protected maleimide appending from different positions (see structures in Table 2). Prot. Mal. = 2,5-dimethylfuran-protected *N*-substituted maleimide; TFA = trifluoroacetic acid.

As we intended to conduct the cycloadditions in an aqueous medium, solid supports made up of polystyrene and polyethylene glycol chains seemed the most convenient for this study since they swell in water, and nowadays are standard resins for the solid-phase assembly of peptides and PNAs. Therefore, NovaSyn TGR and TentaGel resins with the Rink amide-type functionality were used (see Experimental Section).

All PNAs and peptides were assembled on these resins using the standard Fmoc-based methodology (Fmoc/Bhoc monomers in the case of PNAs[46] and Fmoc/*t*Bu in the case of peptides;[47] Bhoc = benzhydryloxycarbonyl). Then the resin-linked polyamides were derivatized with either (*E*)-4,6-heptadienoic acid or 3-maleimidopropanoic acid depending on whether they were intended to remain resin attached or be used as the soluble maleimide-containing component in the DA reaction, respectively.

The outcome of conjugation reactions was examined by HPLC analysis of the crude, and the major products were collected and identified by MALDI-TOF MS. No attempt was made to separate the isomers resulting from the cycloaddition, which, as expected for the size of the molecules, in most cases coelute (see HPLC traces at the Supporting Information). As stated above, both the thiol–maleimide Michael-type addition and the Cu-free Huisgen cycloaddition yield mixtures of isomers that are used as such. Isolation and testing of one of the isomers may become an important issue for biomedical applications but not at this preliminary level. Yields of conjugates (Tables 1 and 2) were calculated as the ratio of the area of the conjugate peak to the sum of the areas of all peaks. Since impurities generated during the solid-phase polyamide assembly also contribute to the total area, reported yields are likely lower than the actual conjugation yields. In reactions with protected peptide–resins it is convenient to determine conjugation yields from the areas of the peaks as appearing on HPLC traces recorded at 220 nm, since only at this wavelength the unreacted peptide can be observed and quantified. On the contrary, for conjugations with PNA–resins 260 nm is the wavelength of choice. In case conjugates were isolated, the isolation yield was determined on the basis of the loading of Fmoc–polyamide–resin prior to incorporation of the diene and accounts for the overall conjugation, deprotection, and purification yield.

On-Resin DA Reactions Involving Water-Soluble Maleimides. The initial set of experiments had different aims: first, to confirm that water could be a suitable solvent for the cycloaddition; second, to identify the mildest possible

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Table 1. Results of the on-Resin Conjugations between Maleimides and Diene−Polyamides

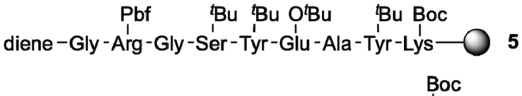
Structures of the reagents*:

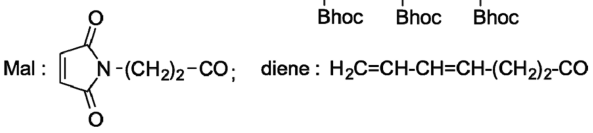
Mal-t-t-Lys-NH_2 **1**

Mal-g-t-t-t-c-Lys-NH_2 **2**

Mal-Gly-Arg-Gly-Ser-Tyr-Glu-Ala-Tyr-Lys-NH_2 **3**

Mal-Ala-Lys-Tyr-Gly-NH_2 **4**



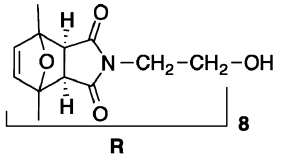


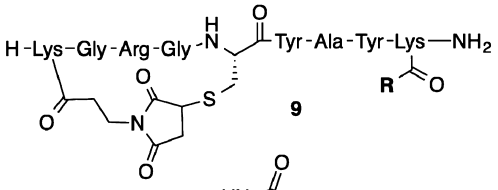
entry	reagents	equiv of Mal.	solvent	[Mal.] (mM)	time (h)	temp. (°C)	yield (%)
1	**1** + **5**	1.5	H_2O	20	70	40	74
2	**1** + **5**	1.5	1:1 H_2O/DMSO	20	70	40	48
3	**1** + **5**	1.5	H_2O	20	24	65	62
4	**1** + **5**	3	H_2O	40	24	65	79
5	**2** + **5**	3	H_2O	40	24	65	69
6	**3** + **6**	3	H_2O	40	24	65	92
7	**4** + 7	5	H_2O	40	24	65	72

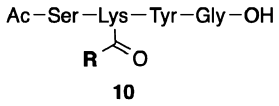
*a,c,g,t = PNA residues with appending adenine, cytosine, guanine, and thymine, respectively; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

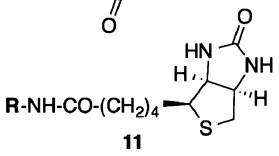
Table 2. Results of the on-Resin Reactions Involving Protected Maleimides and Diene−Polyamides

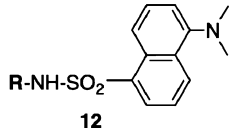
Structures of the reagents (see also table 1):











entry	reagents	equiv of [Prot. Mal.][b]	solvent	[Prot. Mal.] (mM)	time (h)	temp. (°C)	yield[a] (%)	isolated yield (%)
1	**8** + **5**	3	H_2O	40	24	65	82	
2	**9** + **7a**	5	H_2O	40	24	65	58	28
3	**10** + **7a**	5	H_2O	40	24	65	55	30
4	**11** + **5**	3	H_2O	40	24	65	67	
5	**11** + 7	5	H_2O	40	24	65	n.d.[c]	
6	**11** + **7a**	5	H_2O	40	24	65	60	
7	**12** + **5**	3	65:35 H_2O/DMSO	40	24	65	88	48
8	**12** + **7a**	5	65:35 H_2O/DMSO	40	24	65	72	19

[a]Conjugation yields are HPLC based. [b]Prot. Mal. = 2,5-dimethylfuran-protected maleimide-containing reagent (see also Table 1). [c]Not determined: complex HPLC trace (see Supporting Information, page S21).

conditions for the conjugation reaction allowing one to use a minimum excess of the maleimido-derivatized compound and not requiring extremely long reaction times. The conditions reaching the best balance between these variables would be chosen as our standard protocol for reactions in water (Scheme 2a). Table 1 assembles the results of these experiments, in which water-soluble maleimido−PNA **1** and resin-linked, diene-derivatized peptide **5** were chosen as model compounds.

The first two experiments (entries 1 and 2) showed that the reaction between a water-soluble dienophile and a hydrophobic immobilized diene takes place in a higher extent in water than in a water−organic solvent (DMSO = dimethyl sulfoxide) mixture and confirm the favorable effect of water even in these heterogeneous reaction conditions.

The next step was finding suitable conditions to avoid the long conjugation time used in the first experiments. Different combinations of temperature, time, concentration, and equivalents of the maleimide−PNA **1** were tested (Table 1, entries 3 and 4), from which we concluded that the yield of the reaction with 3 equiv of **1** (40 mM concentration) for 24 h at 65 °C was very satisfactory. Although the reaction time was somewhat long, reaction conditions were fairly mild and it was not necessary to use a great excess of maleimido−PNA.

With these results in hand, we decided to further explore the possibilities of the methodology (entries 5−7). The same

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peptide−resin (**5**) was reacted with a longer PNA (**2**), and soluble maleimido−peptides (**3**, **4**) were reacted with diene-derivatized, resin-linked PNAs (**6**, **7**[48]).

It was observed that an increase in the length of the reagents (either the soluble or the immobilized moiety) had a negative impact on the extent of the conjugation reaction, which could be offset by slightly increasing the excess of soluble maleimide.

Use of Protected Maleimides To Obtain Conjugates with Different Linking Sites. It has been described that differently linked conjugates may have different biological effects.[49−52] Standard solid-phase stepwise synthesis may provide hybrid polyamides, either peptide−PNA or PNA−peptide, where the *C*-terminus of one component is linked, either directly or by means of an additional linker, to the *N*-terminus of the other. The experiments described here so far furnish conjugates in which the *N*-termini of the two polyamides are linked through the cycloadduct resulting from the DA reaction and thus give access to different hybrid polyamides.

As stated above, maleimides can be protected with 2,5-dimethylfuran,[38] which allows maleimides to be placed at any position of peptide chains assembled using Fmoc−amino acids.[43] We also described that maleimide deprotection and maleimide-involving click reactions (Diels−Alder, Michael type) can take place simultaneously.[43] If the one-pot deprotection + conjugation reaction could also be performed on a solid support, the scope of the on-resin conjugation via DA cycloaddition might be enlarged, making it possible to link the diene not only to the end of another chain but also to internal positions. This would generate "branched" or "T-shaped" conjugates otherwise difficult to prepare.

To examine whether this alternative was feasible (Scheme 2b), (2,5-dimethylfuran)-protected 2-hydroxyethylmaleimide[38] (**8**) was reacted with peptide−resin **5** (entry 1 of Table 2). HPLC analysis of the crude showed that the target conjugate was formed in 82% yield when using the same reaction conditions as for entries 4−6 of Table 1.

This good result prompted us to tackle the synthesis of more challenging conjugates, either involving more complex reagents or with the aim of preparing conjugates with different linking sites. Cyclic peptide **9**,[53] with a *C*-terminal protected-maleimide (shown in Table 2 as **R**), was reacted with **7a** (see below) to yield a conjugate in which the *C*-terminal residue of a cyclic peptide was linked to the *N*-terminus of a PNA. This reaction proceeded in 58% yield, and the conjugate was isolated in 28% yield (Table 2, entry 2). Then peptide **10**, with a protected maleimide in the middle of the peptide chain, was also reacted with **7a** to render the desired "branched/T-shaped" conjugate also in good yield (Table 2, entry 3). On the basis of these results, we can conclude that the use of [protected-maleimido]−peptides allows synthesizing conjugates with different linkage patterns.

On-Resin Conjugation Reactions Involving Reagents Sparingly Soluble in Water. We also wondered whether molecules sparingly soluble or even nonsoluble in water could be employed in this methodology. To answer this question, two reporter molecules incorporating protected maleimides, biotin and the dansyl fluorophore, were chosen for the first assays. [Protected-maleimide]−biotin **11** is not soluble in water at room temperature but can be solubilized by heating at 150 °C for a few seconds, with no precipitation after cooling. Reaction of **11** with **5** proceeded in 67% yield (Table 2, entry 4), which showed that sparingly soluble molecules could also be used in this conjugation methodology. When **11** was reacted with immobilized diene-[protected-PNA]−resin **7** (Table 2, entry 5), a complex crude was obtained (see Supporting Information, page S21) and the conjugation yield could not be determined. We surmised that the yield was not good and decided to evaluate whether the introduction of a short spacer (one glycine) between the protected PNA chain and the diene allowed the cycloaddition yield to increase (Table 2, entry 6). The yield of the conjugation reaction reached 60%, and the conjugation crude was cleaner (see Supporting Information, page S22). Hence, moving away the diene reactive point and the hydrophobic polyamide may be useful to increase conjugation yields in difficult cases. Subsequent reactions were carried out with the insoluble dansyl fluorophore **12**, which was reacted with both **5** and **7a** (Table 2, entries 7 and 8). In this case a 65:35 (v/v) water/DMSO mixture had to be used (in which **12** was still sparingly soluble). We were gladly surprised to see that HPLC analysis of the reaction crudes showed rather high conversions, in particular, taking into account that cycloadditions were not being carried out in water but in water/organic solvent mixtures. Both conjugates were purified and isolated in 48% and 19% yield, respectively. Thinking about possible reasons for such good conjugation yield, the **12** + **5** reaction was repeated and the Eppendorf tube containing the mixture of reagents was exposed to UV light (254 nm). We could then see that the fluorophore seemed to be adsorbed onto the resin beads surface (see Supporting Information, page S24), presumably because of hydrophobic interactions between the apolar molecule and the carbon-rich chains of the polymer. This could increase the effective concentration of **12** near the reactive points and likely compensate for the decrease of reaction rate expected for a DA reaction in an organic solvent/water mixture, thus accounting for the good yields. These results demonstrate that the on-resin DA reaction can also be carried out with molecules sparingly soluble and even not soluble in water, either by heating the aqueous suspensions to get saturated solutions or by dissolving the dienophile in mixtures with an organic cosolvent and high water content.

Polyamide Double Conjugates. PNAs interact with complementary sequences in nucleic acids with higher affinity than natural oligonucleotides but do not enter cells.[54] The covalent attachment of cell-penetrating peptides to PNAs allows one to overcome this obstacle, but the fate of the conjugate within cells cannot be monitored unless a suitable tag is attached to the molecule. PNAs linked to both fluorophores and carrier peptides are thus interesting molecules, and we anticipated that the on-resin DA cycloaddition could be a valuable tool for their straightforward synthesis. For this proof of principle experiment, cysteine and the diene were subsequently coupled to a protected peptide−resin (Scheme 3), and the double derivatization was achieved after a DA reaction on the solid support and a Michael-type reaction in solution.

A preliminary experiment was run to assess the stability of the Trt group, typically used to protect the cysteine side chain, under the conditions of the DA cycloaddition. 3-Maleimidopropanoic acid (4 equiv) was incubated in water with Fmoc-Cys(Trt)-Gly-Arg(Pbf)-Gly-Ser(*t*Bu)-Tyr(*t*Bu)-Glu(O*t*Bu)-Ala-Tyr(*t*Bu)-Lys(Boc)-resin for 24 h at 65 °C, which was followed by extensive washing, treatment with a 94:2.5:1:2.5 TFA/H_2O/TIS/EDT mixture for 2 h at rt, and HPLC analysis of the crude (see Supporting Information, page S27). MALDI-

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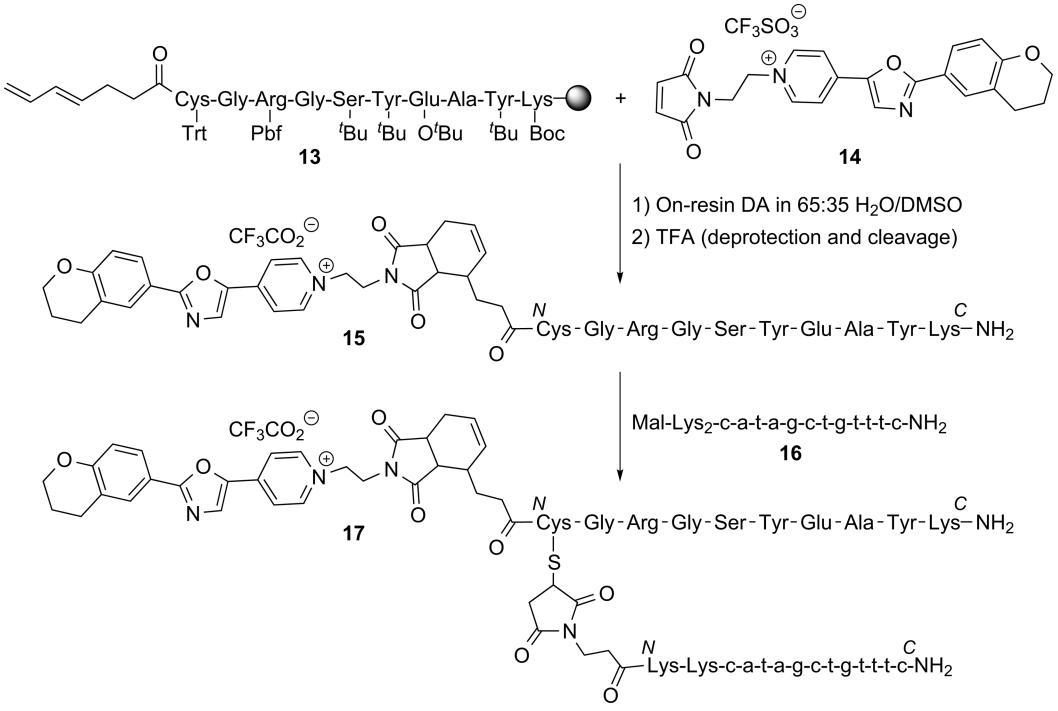
Scheme 3. Double Conjugation Affording Compound 17, in Which a Peptide and a Fluorophore Are Covalently Linked to a PNA[a]



[a]The on-resin DA cycloaddition linked the fluorophore to the diene–peptide, and the solution conjugation reaction (thiol–ene Michael-type reaction) provided the peptide–PNA linkage. Trt = trityl.

TOF mass spectrometric analysis of the major peak confirmed that the Trt group remained stable under the conjugation reaction conditions, since incorporation of 3-maleimidopropanoic acid had not taken place.

Thereafter, the double derivatization was carried out (Scheme 3). More in detail, diene–peptide–resin **13** was prepared by solid-phase assembly of the decapeptide using the standard Fmoc/*t*Bu chemistry and incorporation of (*E*)-4,6-heptadienoic acid. The 1,3-diene-derivatized peptide was then reacted with the commercially available maleimido–fluorophore 1-[2-(maleimido)ethyl]-4-[2-(3,4-dihydro-2*H*-benzopyran-6-yl)-5-oxazolyl]-pyridinium triflate (**14**). As compound **14** was not very soluble in the 65:35 water/DMSO mixture, even less than **12**, a suspension of **14** (3 equiv) was reacted with diene–peptide–resin **13**. After 24 h at 65 °C, the resin-linked conjugate was treated with 94% TFA (in the presence of scavengers, see above) to yield **15**, and the resulting crude was analyzed by HPLC (page S29 of the Supporting Information). The DA reaction yield was 77%. In parallel, two lysine residues and 3-maleimidopropanoic acid were added to the PNA–resin from which **7** had been obtained to yield maleimido–PNA **16** after the final deprotection and HPLC purification. Finally, **15** and **16** (1:1.3 relative molar ratio) were reacted in solution at room temperature, and HPLC analysis of the crude (page S30 of the Supporting Information) showed that in 2.5 h the Michael-type reaction had already gone to completion. Conjugate **17** was purified (23% overall synthesis and isolation yield) and its identity assessed by mass spectrometric analysis.

CONCLUSIONS

In summary, the on-resin DA cycloaddition in water (or high water content mixtures) between a 1,3-diene and a maleimide is a suitable alternative to prepare stable conjugates of polyamides such as peptides and PNAs. The presence of water in the reaction medium allows the reaction to proceed within a reasonable time with no need of heating to very high temperatures (24 h or less at 65 °C). Good to very good conjugation yields can be attained using a small excess of dienophile (1.5–5 equiv), which allows this precious reagent not to be wasted. Yet, this must be balanced by prolonging the reaction time.

On-resin bioconjugations have been also performed using azide–alkyne cycloadditions, most often catalyzed by Cu(I), and experimental conditions vary a lot between the different reports. On the basis of those of three different groups[12,55,56] it can be said that (i) reactions are always carried out in mixtures of water and organic solvents and not pure water, (ii) Cu(I)-catalyzed reactions can satisfactorily take place at 60 °C and in 1 h or less if performed in a MW oven (using 2.3 equiv of soluble reagent)[55] or overnight at room temperature in case activated alkynes are used (4 equiv of soluble reagent was employed in this case),[12] and (iii) copper-free azide–alkyne cycloadditions at room temperature can require from 30 min to 16 h (using 20 equiv of soluble reagent).[56] Therefore, our reaction conditions (excess of soluble reagent, reaction time, temperature) do not substantially exceed those required by the most typical cycloadditions and broaden the scope of possibilities for solid-supported DA cycloadditions. Reaction times could likely be shortened by using more reactive dienes, either cyclic or with more alkyl substituents (or both).[28]

The DA reaction is chemoselective and compatible with PNA and peptide functionalities and their protecting groups and provides a mixture of cycloadducts that remains stable to the acidic treatment that deprotects the polyamide. We never observed any undesired reaction (such as hydrolysis of the

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succinimide ring) on the diene−maleimide cycloadducts, and to our knowledge it has not been described.

The DA cycloaddition can be carried out both with maleimides (many of which are commercially available) and with protected maleimides using aqueous solutions of the dienophile and even suspensions in water/DMSO mixtures. Therefore, this methodology is not limited to hydrophilic dienophiles. Use of protected maleimides broadens the scope of possible targets and allows conjugates with different linking sites to be obtained. In this case, deprotection and conjugation take place simultaneously.

As to the resin-linked reagent, polyamides are assembled on a hydrophilic solid matrix using standard Fmoc-protected building blocks and synthesis procedures, with no sequence restriction. The diene, which can be easily prepared in two steps from cheap starting materials,[57,58] is then incorporated at the *N*-terminus. Since this is followed by conjugation, the diene is never exposed to acids, and the diene−polyamide is not purified. This is an advantage over solution convergent syntheses, where additional time is required to purify the diene-containing moiety prior to conjugation.

Double derivatization of polyamides can be easily carried out by incorporating into the polyamide the diene and a building block containing a functional group allowing for a second click conjugation reaction, such as the Michael-type reaction. Since the first conjugation takes place on the fully protected diene−polyamide, a cysteine residue with a TFA-labile side chain protecting group can be the thiol-providing unit. No thiol−maleimide reaction will occur during the first conjugation because the thiol remains masked, and the second conjugation reaction can be performed after the TFA treatment. In a proof of concept experiment, the on-resin DA cycloaddition was used to attach a fluorophore to a peptide, and a Michael-type reaction in solution linked the fluorophore−peptide conjugate to a maleimido−PNA.

As a modular approach, the methodology here described can easily provide libraries of polyamide conjugates, polyamides derivatized with reporter groups, or both. It is finally worth mentioning that since the DA reactions are conducted in aqueous media, conjugation yields are good enough not to require the addition of metal catalysts, thus precluding the need of assessing their complete absence in the target conjugate.

EXPERIMENTAL SECTION

General Materials and Methods. Acid-free DCM (dichloromethane) was obtained by filtration through basic alumina. Ethyl (*E*)-hepta-4,6-dienoate was synthesized as described in ref 57, (*E*)-hepta-4,6-dienoic acid as described in ref 58, *exo*-(2,5-dimethylfuran)-protected 3-maleimidoethylamine (TFA salt) as described in ref 43, and *exo*-(2,5-dimethylfuran)-protected 3-maleimidoethanol and 3-maleimidopropanoic acid as described in ref 38.

MALDI-TOF mass spectra were recorded using reflector, unless otherwise indicated. The THAP (2,4,6-trihydroxyacetophenone) matrix was prepared by dissolving 10 mg of THAP in 1 mL of a 1:1 (v/v) H_2O/ACN mixture (ACN = acetonitrile). The DHB (2,5-dihydroxybenzoic acid) matrix was prepared by dissolving 10 mg of DHB in 1 mL of a 1:1 (v/v) H_2O/ACN mixture containing 0.1% of TFA.

Polyamide Synthesis. Polyamides were manually assembled in a polyethylene syringe fitted with a polypropylene disk. The DCM used in polyamide synthesis was acid free (passed through a basic alumina column).

Peptide Synthesis. Peptides were assembled on the NovaSyn TGR resin derivatized with a Rink amide linker, which was washed with DCM (3×), DMF (*N*,*N*-dimethylformamide, 3×), MeOH (3×), and DCM (3×). This resin is supplied without an Fmoc protecting group on the amine, so no piperidine treatment is needed prior to chain elongation. Incorporation of the first amino acid onto the resin and peptide assembly were accomplished by using 3 equiv of both Fmoc−amino acid, HOBt·H_2O (1-hydroxybenzotriazole), and DIPC (*N*,*N*′-diisopropylcarbodiimide), all dissolved in a minimal amount of DCM and a few drops of NMP (*N*-methylpyrrolidone), for 90 min at room temperature. Coupling was followed by washing with DCM, DMF, and MeOH (3 × each). In case the coupling was not complete, as assessed by the Kaiser test,[59] it was repeated using 2 equiv of the reagents. The resin was always allowed to swell in DCM for 2 min before Fmoc removal. Removal of the Fmoc groups was effected by reaction with 20% piperidine/DMF (1 × 3 min + 1 × 10 min), followed by washing with DCM (3×), DMF (3×), and DCM (3×). No capping steps were performed, except after incorporation of the last amino acid and prior to incorporation of (*E*)-4,6-heptadienoic acid or 3-maleimidopropanoic acid. The capping step was effected by reaction with Ac_2O/2,6-lutidine/DMF (v/v/v 5:6:89 mixture, 2 × 5 min) and followed by washing with DMF (3×) and DCM (3×). At this level, the substitution degree was determined by taking a carefully weighed aliquot, removing the Fmoc group with piperidine, and quantitating the amount of 9-fluorenylmethylpiperidine formed (λ_{max} = 301 nm, ε = 7800).[60] This value was taken as the substitution degree of the resin-linked diene−polyamides in conjugation experiments (see below), without any further correction for the addition of the diene, and used to determine the overall synthesis and purification yield for the isolated conjugates. Fmoc-Gly-Arg(Pbf)-Gly-Ser(^{t}Bu)-Tyr(^{t}Bu)-Glu(O^{t}Bu)-Ala-Tyr(^{t}Bu)-Lys(Boc)-resin, substitution degree: 0.17 mmol/g.

Activation of 3-maleimidopropanoic acid was carried out with DIPC only (90 min reaction time). Even though activation with DIPC and HOBt·H_2O seems not to cause any harm on the maleimide, use of only the carbodiimide is safer to prevent addition of HOBt to the maleimide. (*E*)-4,6-Heptadienoic acid was coupled in the same conditions as amino acid residues (DIPC + HOBt). Both the diene−acid and 3-maleimidopropanoic acid were coupled to the peptide by using 10 equiv of the appropriate reagents.

Deprotection of *N*-terminal maleimido−peptides and cleavage from the resin was generally carried out by reaction with a v/v/v 95:2.5:2.5 TFA/H_2O/TIS mixture (1−2 mL) for 2 h at room temperature. In case of cysteine-containing peptides (such as conjugate **15**), a v/v/v/v 94:2.5:1:2.5 TFA/H_2O/TIS/EDT mixture (EDT = 1,2-ethanedithiol) was used. Filtrate and washings (TFA, no scavengers) were collected and concentrated by blowing N_2 over the mixture, and diethyl ether was added to the resulting oil or semisolid (3−5 mL). Water was added to dissolve the peptide (2−3 mL), the biphasic mixture was shaken, and the two phases were allowed to separate. The organic phase was discarded, and new diethyl ether was added. This washing procedure was repeated 3 times. The aqueous phase was then lyophilized and the peptide analyzed and purified by HPLC.

N-Terminal diene−peptide−resins were stored at 8 °C until conjugation was effected.

PNA Synthesis. PNAs were assembled on the TentaGel R RAM resin (derivatized with a Rink amide linker). Prior to chain elongation the resin was washed with DCM (3×), DMF (3×), MeOH (3×), and DCM (3×), treated with 20% piperidine/DMF (1 × 3 min + 1 × 15 min), and washed with DCM (3×), DMF (3×), and DCM (3×). Incorporation of the PNA monomers was effected by reaction with 4 equiv of both the Fmoc monomer and COMU (1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate) and 8 equiv of DIPEA (*N*,*N*-diisopropylethylamine), all dissolved in a minimal amount of NMP. This mixture was preactivated for 1 min before being poured onto the resin and allowed to react for 90 min at rt. Coupling was followed by washing with DCM, DMF, and MeOH (3 × each). In case the coupling was not complete, as assessed by the Kaiser test, it was repeated as before. The resin was always allowed to swell in DCM for 2 min before Fmoc removal. Removal of the Fmoc groups was performed by reaction with 20% piperidine/DMF (1 × 3 min + 1 × 10 min), followed by washing with DCM (3×), DMF (3×), and DCM

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(3×). A capping step was carried out after incorporation of each monomer, as described above, to ensure that no trace of free amines was left. After the polyamide chain was elongated, the substitution degree was determined by taking a carefully weighed aliquot, removing the Fmoc group with piperidine and quantitating the amount of 9-fluorenylmethylpiperidine formed (λ_{max} = 301 nm, ε = 7800).[60] This value was taken as the substitution degree of the resin-linked diene−polyamides in conjugation experiments (see below), without any further correction for the addition of the diene (or the diene and one glycine as in **7a**), and used to determine the overall synthesis and purification yield for the isolated conjugates. Fmoc-t-t-Lys(Boc)-resin, substitution degree: 0.17 mmol/g. Fmoc-c(Bhoc)-a(Bhoc)-t-a(Bhoc)-g(Bhoc)-c(Bhoc)-g(Bhoc)-t-t-t-c(Bhoc)-resin, substitution degree: 0.073 mmol/g.

Activation of 3-maleimidopropanoic acid and (*E*)-4,6-heptadienoic acid was effected as for the synthesis of peptides. Storage of the diene−PNA−resins and cleavage of the maleimido−PNAs were also the same as for the peptide−resins.

*exo-(2,5-Dimethylfuran)-Protected Biotin−Maleimide (**11**).* Biotin−NHS ester (NHS = *N*-hydroxysuccinimide) (154 mg, 0.49 mmol) was dissolved in DMF (2 mL), and *exo*-(2,5-dimethylfuran)-protected 3-maleimidoethylamine·TFA (207 mg, 0.59 mmol, 1.2 equiv) was added. After complete dissolution of all products triethylamine (83 μL, 0.59 mmol, 1.2 equiv) was added and the mixture stirred at 40 °C for 18 h. The solvents were evaporated in vacuo, and the crude product was washed twice with DCM (HPLC quality) to obtain a white powder solid (104 mg, 45%).

^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.78 (t, J = 5.9 Hz, 1H), 6.41 (s, 1H), 6.36 (s, 2H), 6.35 (s, 1H), 4.30 (m, 1H), 4.13 (m, 1H), 3.40 (t, J = 6.3 Hz, 2H), 3.16 (q, J = 6.1 Hz, 2H), 3.12−3.06 (m, 1H), 2.86 (s, 2H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 2.57 (d, J = 12.4 Hz, 1H), 1.99 (t, J = 7.4 Hz, 2H), 1.64−1.56 (m, 1H), 1.53 (s, 6H), 1.50−1.39 (m, 3H), 1.35−1.22 ppm (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 174.8, 172.2, 162.7, 140.6, 86.9, 61.0, 59.2, 55.4, 52.2, 39.8, 37.7, 35.9, 35.1, 28.2, 28.0, 25.0, 15.7 ppm. HRMS (ESI, positive mode): M calcd for $C_{22}H_{30}N_4O_5S$ 462.1937, m/z found 463.2000 $[M + H]^+$, 485.1815 $[M + Na]^+$.

*exo-(2,5-Dimethylfuran)-Protected Dansyl−Maleimide (**12**).* Dansyl chloride (31 mg, 0.115 mmol, 1.33 equiv) was dissolved in DMF (6.5 mL), and DIPEA (38 μL, 0.215 mmol, 2.5 equiv) and *exo*-(2,5-dimethylfuran)-protected 3-maleimidoethylamine·TFA (30 mg, 0.086 mmol) were added. The reaction mixture was stirred for 1 h at rt and then taken to dryness in vacuo. DCM (20 mL) was added to dissolve the resulting oil, and this organic phase was washed with 1% aq HCl (3 × 15 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo (33 mg, 82%). The product was used without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.51 (d, J = 8.5 Hz, 1H), 8.21 (dd, J = 12.9, 8.6 Hz, 2H), 7.52 (m, 2H), 7.16 (d, J = 7.5 Hz, 1H), 6.25 (s, 2H), 5.29−5.26 (m, 1H), 3.57−3.51 (m, 2H), 3.13 (q, J = 6.0 Hz, 2H), 2.86 (s, 6H), 2.55 (s, 2H), 1.62 ppm (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 187.2, 175.0, 151.9, 140.7, 134.4, 130.4, 129.8, 129.4, 128.4, 123.3, 119.0, 115.2, 87.6, 52.4, 45.4, 41.1, 38.1, 15.8 ppm (impurities detected at 1.26 ppm in the ^{1}H NMR correlate with impurities at 29.7 and 21.8 ppm as seen by gHSQC). HRMS (ESI, positive mode): M calcd for $C_{24}H_{27}N_3O_5S$ 469.1671, m/z found 470.1745 $[M + H]^+$, 939.3406 $[2M + H]^+$.

Conjugation. General Procedure. The appropriate amount (2−17 mg, see below) of resin-linked, fully protected diene−polyamide (PNA or peptide) was carefully weighed into a 500 μL Eppendorf tube (the μmoles of diene were calculated on the basis of the weigh and substitution degree of the polyamide−resin, see above). The maleimide-containing reactant was dissolved (or suspended in the solvent), poured into the Eppendorf, and allowed to react (solvent, reaction time, temperature, molar excess, and concentration of the reagent for each particular case are indicated in Tables 1 and 2 and summarized also in the Supporting Information, Table S1, page S7). The Eppendorf tube was sonicated immediately after the addition of the dienophile to ensure homogeneity of the mixture (until it was observed that all beads were covered by the maleimide solution) and three or four times over the reaction course. The Eppendorf tube was placed in a sand bath heated at the desired temperature, and the reaction was allowed to proceed with no additional stirring.

After the appropriate reaction time (see Tables 1 and 2), the Eppendorf content was filtered through a 2 mL syringe equipped with a filter, and the resin was washed with H_2O (3×), ACN (3×), DMF (3×), H_2O (3×), ACN (3×), and DCM (3×) and allowed to air dry. In the case of sparingly soluble maleimide-containing reagents (biotin and dansyl derivatives), 0.1% TFA was added to the H_2O and ACN washings to increase their solubilizing properties. After washing the resin, the conjugate was cleaved as described in the polyamide synthesis section, then analyzed by HPLC, and characterized by MALDI-TOF mass spectrometry (see Supporting Information for yields and HPLC profiles of the crudes, section 4). Yields are also indicated in Tables 1 and 2. In some cases (see tables) conjugates were purified (conditions and HPLC traces of purified products are shown in the Supporting Information).

Additional Data for Conjugation Experiments Reported in Tables 1 and 2. **1** + **5** (Table 1, entry 1): 8.5 mg of **5**, 106 μL of H_2O. **1** + **5** (Table 1, entry 2): 8.1 mg of **5**, 101 μL of 1:1 H_2O/DMSO. **1** + **5** (Table 1, entry 3): 6.7 mg of **5**, 83 μL of H_2O. **1** + **5** (Table 1, entry 4): 5.6 mg of **5**, 70 μL of H_2O. **2** + **5** (Table 1, entry 5): 7.7 mg of **5**, 97 μL of H_2O. **3** + **6** (Table 1, entry 6): 2.8 mg of **6**, 36 μL of H_2O. **4** + **7** (Table 1, entry 7): 5.1 mg of **7**, 47 μL of H_2O. **8** + **5** (Table 2, entry 1): 6.1 mg of **5**, 80 μL of H_2O. **9** + **7a** (Table 2, entry 2): 16.5 mg of **7a**, 151 μL of H_2O. **10** + **7a** (Table 2, entry 3): 2.7 mg of **7a**, 25 μL of H_2O. **11** + **5** (Table 2, entry 4): 5.3 mg of **5**, 66 μL of H_2O. **11** + **7** (Table 2, entry 5): 5.6 mg of **7**, 51 μL of H_2O. **11** + **7a** (Table 2, entry 6): 7.4 mg of **7a**, 67 μL of H_2O. **12** + **5** (Table 2, entry 7): 12.0 mg of **5**, 150 μL of H_2O. **12** + **7a** (Table 2, entry 8): 16.5 mg of **7a**, 150 μL of H_2O.

Double-Conjugation Experiments (Scheme 3). **13** + **14**: Commercially available fluorophore **14** (3.7 mg, 6 μmol, taking into account that it was 90% pure) was suspended in 150 μL of a 65:35 (v/v) H_2O/DMSO mixture, and this suspension was poured into an Eppendorf tube containing diene−peptide−resin **13** (12.0 mg, 2 μmol of diene−peptide). The cycloaddition reaction was carried out at 65 °C for 24 h, after which time the resin was filtered and washed as indicated above. Deprotection and cleavage was performed by reaction with a 94:2.5:1:2.5 mixture of TFA/H_2O/TIS/EDT for 2 h at room temperature, and filtrates and washings were collected and treated as indicated above (peptide synthesis section) to yield crude **15**. Crude **15** was purified by HPLC and characterized by MALDI-TOF MS.

15 + **16**: Maleimido−PNA **16** (1 μmol, 1.3 equiv) and compound **15** (0.8 μmol) were reacted in H_2O (200 μL; maleimido−PNA concentration: 4 mM) for 2.5 h at 37 °C. The target conjugate (**17**) was purified by HPLC (0.47 μmol of pure **17** was isolated) and characterized by MALDI-TOF MS (see Supporting Information). Overall yield (from Fmoc−peptide−resin, peptide derivatization with the diene, two conjugation reactions, and two purification steps): 23%.

■ ASSOCIATED CONTENT

Supporting Information

HPLC and mass spectrometric characterization data of the different compounds (polyamides and conjugates), assessment of the diene stability to polyamide deprotection conditions, results of the conjugation assays, and ^{1}H and ^{13}C NMR spectra of compounds **11** and **12**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00592.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: anna.grandas@ub.edu

Notes

The authors declare no competing financial interest.

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