

Scope and Limitations of Boron Fluorescent Complexes from Stable Nitrile Oxides in ABPP Assays

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Supporting Information

ABSTRACT: Anthracenenitrile oxide undergoes 1,3-dipolar cycloaddition reactions with 1-substituted-4-(prop-2-yn-1-yloxy)benzene affording the expected isoxazoles in good yields and as single regioisomers. N–O Bond cleavage and boron complexation afforded the corresponding boron complexes, derivatized with either a triple bond for click-chemistry applications or an oxime group for nitrile oxide 1,3-dipolar cycloaddition. The optical properties of these compounds show potential for employment as fluorescent tags in imaging techniques. The activity-based protein profiling assays showed good reactivity for the synthesized probes even with short peptide chains (epoxomicin). Scope and limitations are discussed in the light of the obtained results.

INTRODUCTION

1,3-Dipolar cycloadditions can be undoubtedly considered a pillar of the synthesis of isoxazoles. The versatility of this strategy was recently applied to synthesize structures designed for imaging. Albeit the number of works devoted to this topic is increasing, the applications of the probes constructed via this pathway remain quite low so far.¹ In a recent conceptual work, we successfully employed 1,3-dipolar cycloaddition of stable aromatic nitrile oxides to afford novel fluorescent compounds. The reported strategy is the cornerstone to furnish boron-substituted complexes suitable for biochemical applications; the nitrile oxide-based protocol is cleaner and selective with dipolarophiles and can be a useful alternative to the use of azides. Indeed, once properly derivatized, it can find proper use in the activity-based protein profiling (ABPP).²

ABPP is one of the most powerful tools to gain insight into complex biological systems, e.g., the activity of enzymes in complex proteasomes.³ The aim of ABPP resides in the visualization of the active forms of the enzymes using chemical probes directed to the active site of a target protein, resulting in the selective labeling of the sole catalytically active form of the enzyme.⁴

Structurally, chemical probes consist of three different parts: recognition tag, variable length linker, and warhead (ligation handles) containing the functional groups to link the probe with the target substrate with highly specific interactions that make the probe selective for a well-defined biological structure (Scheme 1). The ligation strategy we wish to exploit is based on 1,3-dipolar cycloaddition for the two-step activity-based

labeling of endogenously expressed enzymes in complex biological samples.⁵

The investigated probes aimed to be applied in a labeling procedure that enabled us to label active proteasome β -subunits selectively in cellular extracts and in living cells.

In our previous work, we detailed the synthetic strategy and the fluorescence study of compound **5** that structurally follows the aforementioned paradigms. The compound is synthesized starting from a 1,3-dipolar cycloaddition reaction between 1-iodo-4-(prop-2-yn-1-yloxy)benzene (**2**), affording the 5-substituted isoxazole **3** in very good yields as a single regioisomer. The outcome of the transformation nicely follows the predictions based on the frontier orbital theory.² Cleavage of the N–O bond and BF_3 complexation furnish the corresponding fluorescent boron complex of type **5** (Scheme 1). The molecule bears an anthryl substituent (Ar = 9-anthryl) on the tag and a triple $\text{C}\equiv\text{C}$ bond on the warhead end terminus (R = $-\text{C}\equiv\text{CH}$) of the probe, suitable for click-chemistry applications and late-stage functionalization.

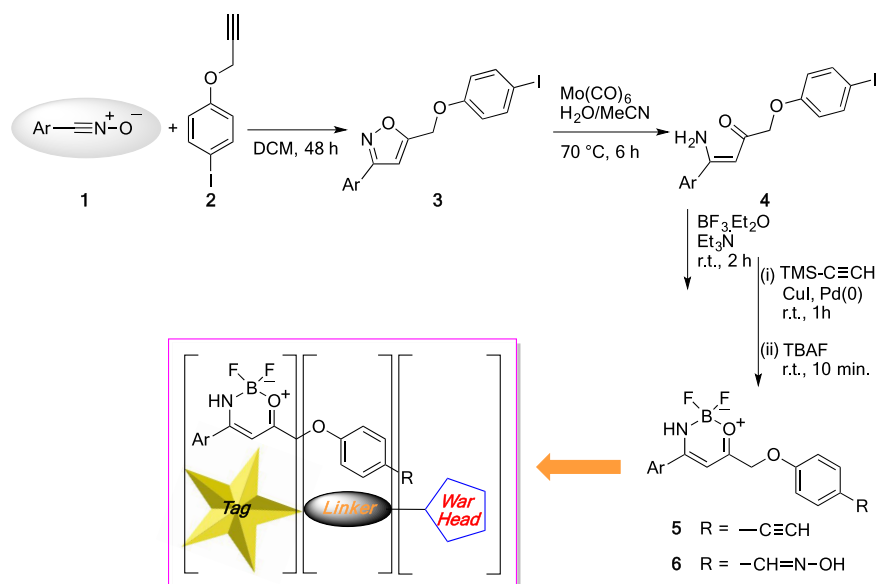
Here, we present a strategy to prepare chemical probes that maintain the same tag structure, while bearing variable ligation handles prone to orthogonal functionalization with a proteasome inhibitor peptide. In compound **5**, the triple bond requires the use of a substrate bearing a dipole (typically an azido-derivative) to connect the two sides of the chemical reporter (Scheme 2). The newly designed compound **6** bears

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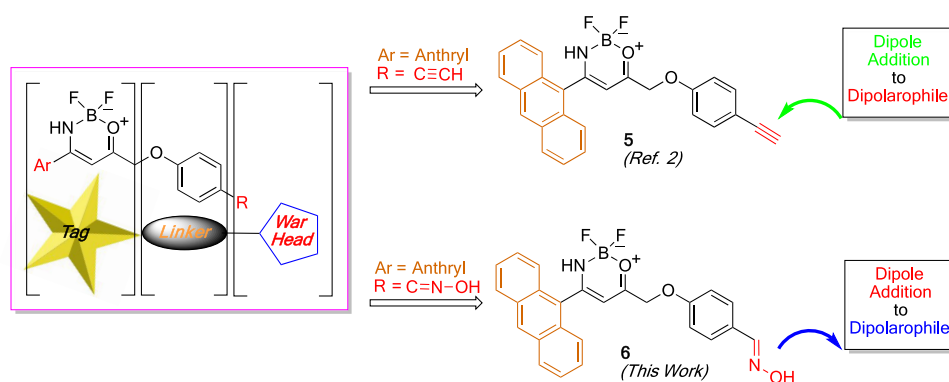
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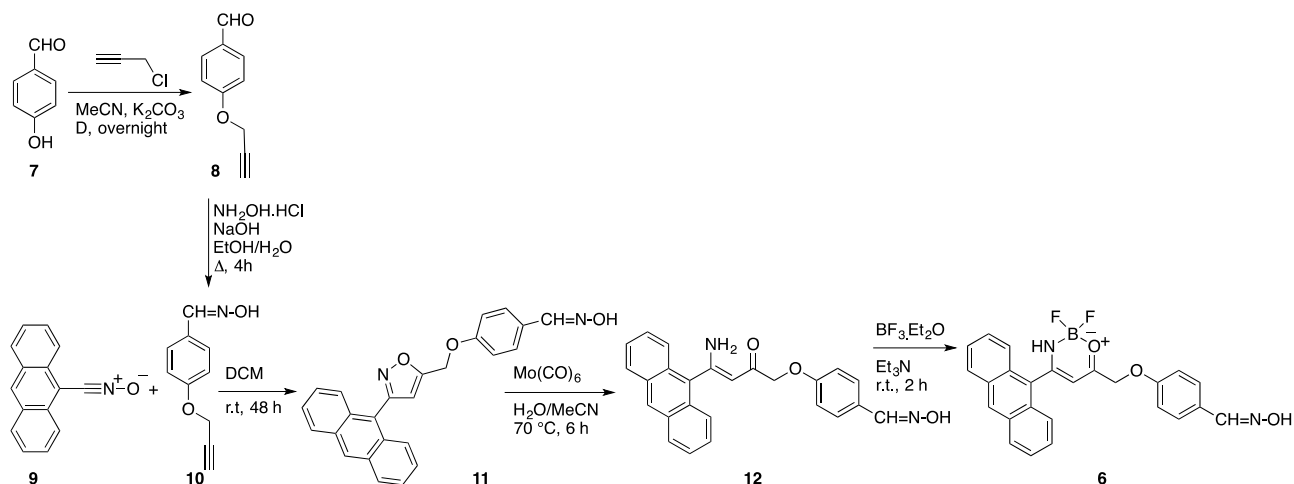
Scheme 1. From Nitrile Oxides 1 to 5-Substituted Isoxazoles 3, N–O Bond Cleavage and Boron Complexation: Synthetic Route to Fluorescent Probes of Type 5 and 6



Scheme 2. Probe Structures: Dipolarophile and Dipole Precursor Ligation Handle Structures



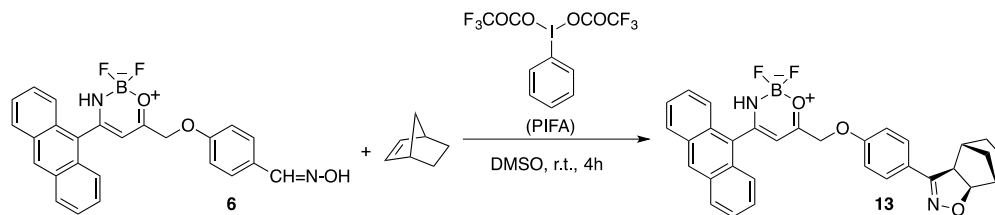
Scheme 3. Probe Synthesis: From 4-Hydroxybenzaldehyde (7) to 4-((4-(Anthracen-9-yl)-2,2-difluoro-2,3-dihydro-1λ³,3,2λ⁴-oxazaborinin-6-yl)methoxy)benzaldehyde Oxime (6)



an oxime moiety on the warhead part that is suitable to be oxidized to nitrile oxide. Hence, the probe is another 1,3-dipole that can be attached to a double (or triple) bond located on a target substrate. One of the great advantages of the use of

nitrile oxides is the fact that the cycloaddition reactions are cleaner than those in the presence of azides; these latter ones require metals (copper ions, typically) that are detrimental for the cellular environment.

Scheme 4. 1,3-Dipolar Cycloaddition Reaction of Probe 6 with Norbornene



The two chemical probes 5 and 6 will be compared from the photophysical point of view and tested by coupling them with differently functionalized epoxomicin derivatives. Ultimately, competitive ABPP assays will be performed to verify the maintenance of proteasome inhibitor properties and possible differences in terms of selectivity.

RESULTS AND DISCUSSION

The commercially available 4-hydroxybenzaldehyde 7 was derivatized with freshly distilled propargyl chloride in the presence of a base under reflux in acetonitrile (Scheme 3). The ethynyl derivative 8 is a known compound⁶ and was transformed into the corresponding oxime by treatment with hydroxylamine in a hydro-alcoholic solution, to afford compound 10.⁷ The oxime dipolarophile was allowed to react with an equimolar amount of anthracenenitrile oxide 9 in anhydrous dichloromethane (DCM) at room temperature for 48 h. After purification, the 5-substituted isoxazole 11 was obtained in 66% yield.⁸ In the ¹H NMR spectrum (dimethyl sulfoxide, DMSO), the diagnostic signal of the H4 proton of the isoxazole ring was detected at δ 7.03 ppm. The inclusion of the dipolarophile moiety into the cycloadduct was also confirmed by the presence of the methyleneoxy group at δ 5.53 ppm (singlet) and by the highly deshielded oxime OH signal at δ 11.06 ppm.

The selective N–O bond cleavage⁹ in the isoxazole moieties was performed using Mo(CO)₆ to give the 4-[(4-amino-4-(anthracen-9-yl)-2-oxobut-3-en-1-yl)oxy] benzaldehyde oxime 12 as a reddish solid in 68% yield. As previously reported,² the signals detected in the ¹H NMR spectrum denote the existence of an intramolecular hydrogen bond between the amino group and the carbonyl functionality obtained from the N–O bond cleavage. As a consequence, one of the NH₂ protons is found at δ 8.47 ppm whereas the other is strongly deshielded at δ 10.25 ppm. The latter is the direct evidence of the 6-membered cyclic array kept together by the hydrogen bond.

The enaminketone 12 was used as ligand for boron complexation,¹⁰ achieved by adding BF₃·Et₂O in an anhydrous DCM solution, in the presence of Et₃N (Scheme 3). Boron complex 6 was obtained as a yellow solid in 40% yield. In the ¹H NMR spectrum, a single NH proton signal is detected at δ 7.62 ppm, as a consequence of the newly formed bond between the nitrogen and the boron.

To verify the reactivity of the fluorescent probe 6 as 1,3-dipole, we oxidized the oxime group with ([bis(trifluoroacetoxy)iodo]benzene) (PIFA) in the presence of an excess of norbornene (Scheme 4).¹¹ Norbornene, an excellent trap for nitrile oxides,¹² is able to attach the in situ formed nitrile oxide from compound 6, affording the corresponding 1,3-dipolar cycloadduct 13 in 81% yield, and the reaction does not require any metal promoter such as copper that is normally used with azides. Hence, the reaction is cleaner, and after simple chromatographic purification,

compound 13 was fully characterized; the presence in the ¹H NMR spectrum of the signals corresponding to the isoxazole protons at δ 3.64 and 4.55 ppm clearly indicates the successful coupling between the nitrile oxide moiety and the C=C double bond of the norbornene.

To evaluate the potential use of the new boron complex 6 as a chemical probe in biological studies, we performed some UV–vis and fluorescence studies in solvents of different polarities, comparing the results with those previously obtained for compound 5.²

Figure 1 shows the UV–vis spectra of compounds 5 and 6 in DCM as solvent. The oxime derivative 6 showed an absorption

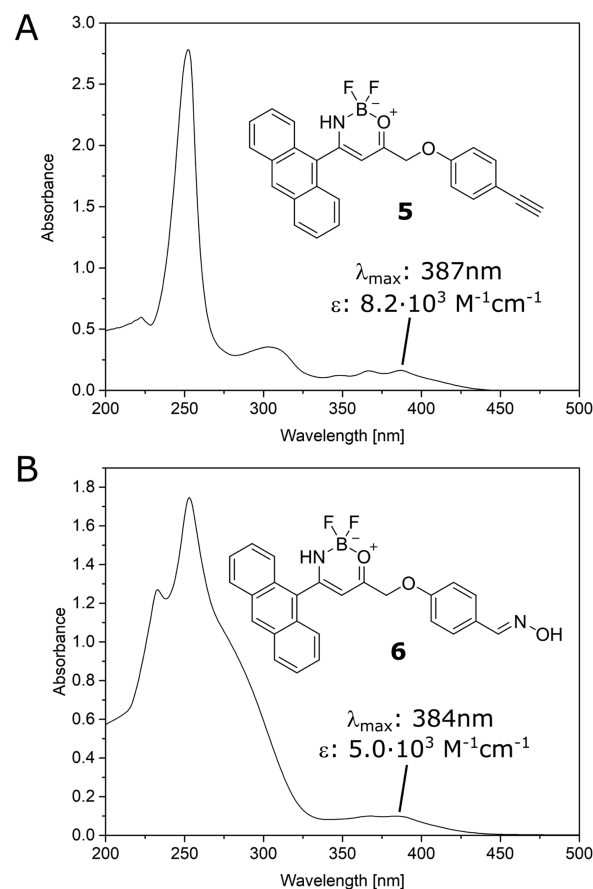


Figure 1. UV–vis spectra of compounds 5 (A) and 6 (B) in DCM (2×10^{-5} M solutions).

band at 384 nm ($\epsilon = 5.00 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) with a shoulder at 238 nm and vibrational peaks. Analogously, the BF₂ complex 5 shows an absorption band at 387 nm ($\epsilon = 8.20 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) along with more structured vibrational peaks compared with compound 6. The change in functional groups on the phenyl ring does not drastically shift the absorption peak in the

near-UV region. It however results in a broadening of the absorption band around 250 nm that merges with the blue-shifted shoulder.

The fluorescence emission spectra of **5** and **6** are shown in Figure 2. The fluorescence maxima for both the compounds

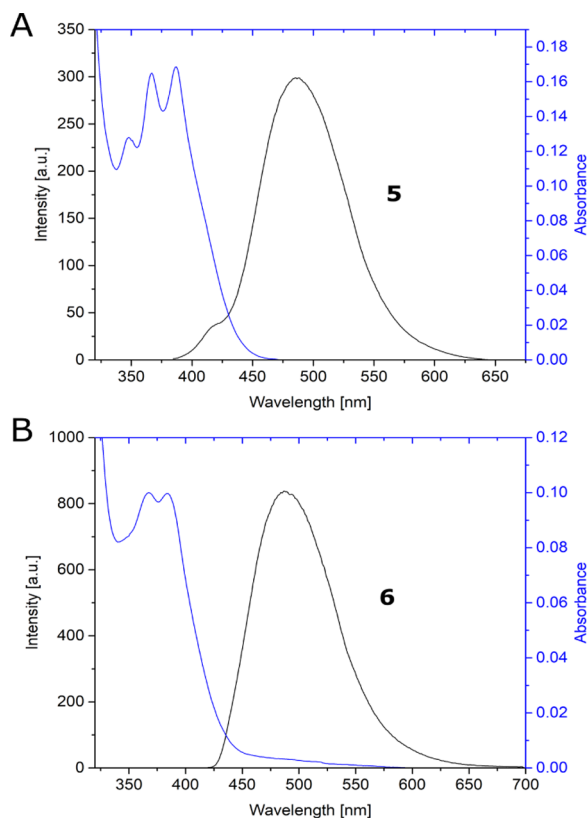


Figure 2. Fluorescence spectra of compounds **5** (A) and **6** (B) in DCM (blue line, absorption; black line, emission).

appear at 490 nm, indicating a Stokes' shift of ca. 5630 cm^{-1} for the two complexes. The emission spectra are structureless and present a single intense peak. The complete loss of the vibrational features, along with the considerable Stokes' shift, could be indicative of a structural rearrangement occurring at the excited state. This feature is less pronounced in more rigid fluorescent boron derivatives.¹³ Indeed, the first excited-state optimized structure of compound **6** at the SMD(DCM)-TD-CAM-B3LYP/6-31G(d,p)//TD-B3LYP/6-31G(d,p) level of theory differs from the Franck–Condon one, due to the torsion along the bond connecting the boracycle to the anthryl moiety.¹⁴ The associated dihedral angle varies from ca. 66 to 41° . Such a structural change is an indication of the nature of the electronic transition associated to fluorescence. The excited state populates an antibonding orbital that spans from the anthryl moiety to the boracycle with an electron coming from the anthracene (see Figure 3). Consequently, the two connected cycles involved in the transition bend to increase the conjugation in the molecule.

In DCM, compound **6** is characterized by fluorescence quantum yield $\Phi_F = 0.07$ whereas compound **5** exhibits $\Phi_F = 0.60^2$ (both values were determined relative to the fluorescent intensity of a boron-substituted reference compound; for further details refer to the Supporting Information SI).¹⁰ The low quantum yields of compound **6** even in DCM could be the indication of intermolecular quenching of fluorescence. It is

known that a protic environment can influence the excited-state nature of fluorophores.¹⁵ The source of this interaction could be the hydroxylamino derivative in compound **6**, absent in compound **5**. To test our hypothesis, we optimized compound **6** at the SMD(DCM)-TD-CAM-B3LYP/6-31G(d,p)//TD-B3LYP/6-31G(d,p) level, in the presence of a water molecule, as a prototypical protic compound. In this case, the excited-state changes in nature and a charge-transfer electron transfer from the phenoxy moiety to the anthryl one quenches the fluorescence. Hence, we can conclude that the interaction of **6** with a protic solvent or with its hydroxylamino moiety can be the culprit for the low quantum yields of **6** (see Figure 3).

The solvent effect on the absorption and fluorescence properties of the new boron complex **6** was examined (spectra reported in the SI), and Table 1 reports the Φ_F values obtained. The absorption (λ_{max}) values of **6** are not affected by solvent polarity, suggesting that the dipole moments of the molecule in the ground and excited states are almost the same.¹⁶ On the other hand, the maximum of fluorescence emission (λ_{em}) values showed a blue shift of about 40 nm in polar solvents with respect to the value in DCM. This determined a Stokes' shift reduction at just about ca. 3820 cm^{-1} . These results show the same trend as that observed for compound **5**.² Significantly, the Φ_F values dramatically diminish upon polarity changes as well as viscosity; remarkable drops in quantum yields are observed in MeOH (0.01) and in ethylene glycol (0.02). The same happens in the 1:1 DMSO/H₂O mixture with Φ_F value = 0.01. This could suggest some drawbacks when the compound is used in a biological environment.¹⁷

On the basis of novel and previous observations and with the aim to employ compounds **5** and **6** in ABPP assays, we performed a series of coupling experiments with suitable epoxomicin derivatives. Azido-substituted epoxomicin **14**, a proteasome inhibitor peptide, was coupled with the ethynyl-probe **5** under standard click-reaction conditions (Scheme 5).^{3,4} The expected product **15** was isolated and purified by column chromatography. Retaining of water in the final compound required a prolonged drying step. After the drying process, the product was obtained in 55% yield and the mass spectrum (MS) reported a mass value of 1023.30 m/z corresponding to the molecular weight of **15** (1006.94 m/z) plus OH (MW + OH). This discrepancy in the MS spectra values can be tentatively attributed to the harsh purification protocol followed (even with standard methods) that presumably left on the polar peptide chain residues of the basic treatment. We cannot exclude the epoxide ring opening to give a diol or even the stabilization of the hydrate forms of the carbonyl group adjacent to the oxirane ring.

On the other hand, norbornene-substituted epoxomicin **16** was allowed to react with oxime **6** under oxidative conditions (using phenyliodine diacetate (PIDA) as an oxidant in dimethylformamide (DMF) at room temperature). The in situ-generated nitrile oxide of **6** was added exo-selectively¹⁹ to the C=C bond of the norbornene moiety to afford the corresponding 1,3-dipolar cycloadduct **17** (Scheme 6), presumably as a mixture of regioisomers that would not affect the biological behavior. Due to the known reactivity of nitrile oxides with nucleophilic centers and the nature of the dipolarophile, the purification step was quite problematic and several steps of chromatographic purification were needed. Nevertheless, the final cycloadduct was obtained in 45% yield

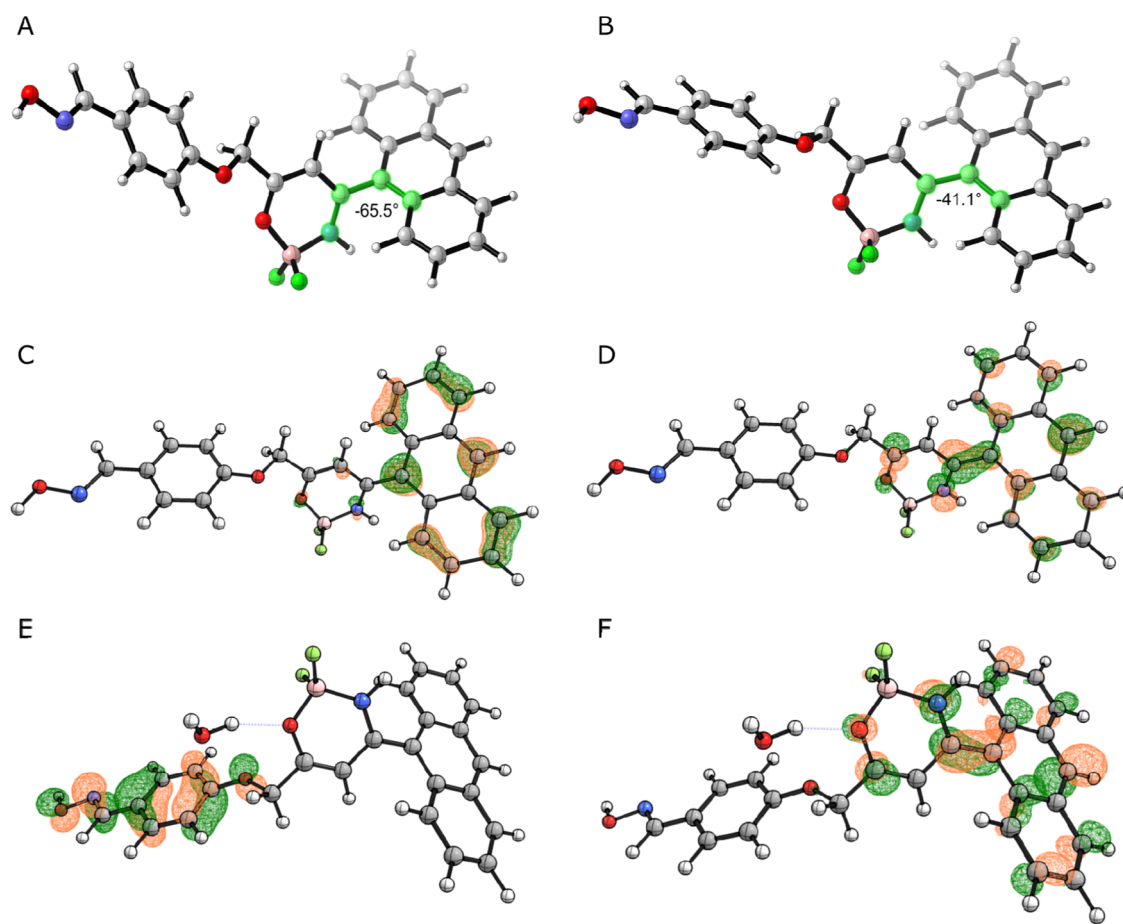


Figure 3. Optimized structure of the ground state of compound **6** (A) and its first excited state (B). Orbitals involved in the first excited state of **6** without (C, D) and with (E, F) protic interactions taken into account. All calculations are at the SMD(DCM)-(TD)-CAM-B3LYP/6-31G(d,p)//(TD)-B3LYP/6-31G(d,p) level, and the structures correspond to energy minima.

Table 1. Absorption λ_{max} (nm) and Fluorescence Quantum Yield (Φ_{F}) Values for Complex **6** in the Listed Solvents^a

solvent	dielectric constant	viscosity ¹⁸ (cP at 20 °C)	λ_{max} (nm)	λ_{em} (nm)	Φ_{F}
DCM	8.9	0.44	384	490	0.07
MeOH	33	0.55	380	488	0.01
ethylene glycol	39	23.5	384	455	0.02
DMSO/H ₂ O	55	3.30	384	450	0.01

^aConcentration 2×10^{-5} M in all solvents.

and the MS spectrum confirmed the structure as drawn in Scheme 6 with MW = 1199.30 (theor. 1199.15) *m/z*.

The coupling products **15** and **17** were then tested in competitive ABPP assays (sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE) to check the maintenance of the proteasome inhibitor properties of epoxomicin²⁰ and the display of fluorescence properties. The competitive SDS-PAGE assay was performed with some other marked proteasome inhibitors (details in the SI).

The analysis was done on Raji cell lysate (1 μL), containing both immuno and constitutive proteasome ([proteasome] = 20 $\mu\text{g } \mu\text{L}^{-1}$). Different samples of lysate were incubated for half an hour at 37 °C along with 9 μL of 26S assay buffer and 1 μL of a DMSO solution of compounds **15** or **17** at different concentrations (0, 0.1, 1, 10, and 100 μM). Then, 1 μL of the 10 \times ABP cocktail was added and the samples were incubated for other 30 min. Finally, 5 μL of the 4 \times sample buffer to

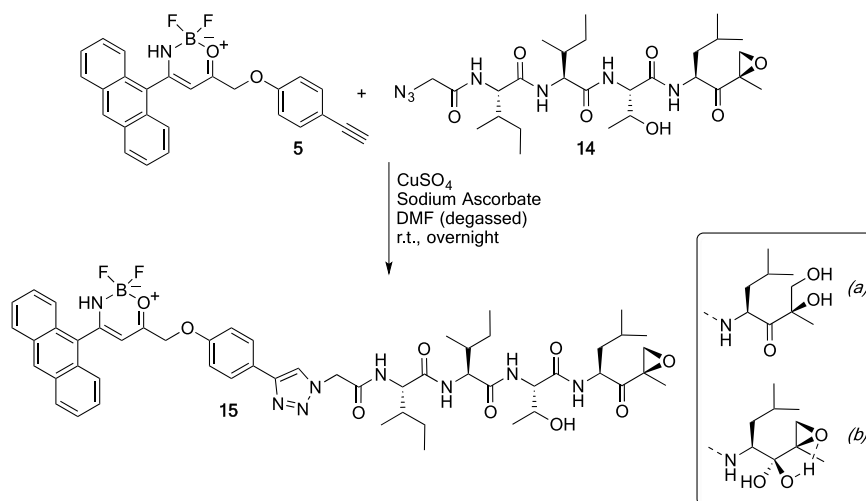
denature and reduce proteins was added and the samples were boiled at 100 °C for 5 min.

The samples were fixed in the different lanes of a SDS-PAGE stacking gel and then were made to run by gel electrophoresis on the running gel. Figure 4 shows the SDS-PAGE gel results where in the lane 1, it is possible to see the different β subunits of the proteasome, highlighted with specific fluorescent markers that bind them selectively: in blue, Cy5-NC-001 linked to the $\beta 1$ subunits, in green, BODIPY(FL)-LU-112 was selective to the $\beta 2$ ones, and in red, BODIPY(TMR)-NC-005-VS linked to the $\beta 5$ ones.

In lane 2, 3, 4, and 5, increasing concentrations of probe **15** (from 10 to 10⁴ nM) are reported, whereas in lane 7, 8, 9, and 10, the same increasing concentration of probe **17** (from 10 to 10⁴ nM) can be found. For a concentration of 1000 nM, both probes show a very good competition in binding the proteasome; in fact, in lanes 4 and 5 and 9 and 10 the colored lines relative to the other marked inhibitors are not visible. In lane 8, the red stripes relative to the $\beta 5$ subunits disappeared, suggesting a higher activity of the probe **17** for these subunits.

To detect the fluorescence of probes **15** and **17** while they bind the proteasome, several different light sources were tried to excite the fluorophores but unluckily they did not match the excitation wavelength of our probes and no fluorescence were registered. Some possible explanations can be considered. We cannot state if these probes maintain their fluorescence during the interaction with the proteasome; it is also possible that the

Scheme 5. Coupling of Ethynyl-Probe 5 with Azido-Substituted Epoxomicin 14; Inset Shows the Possible Structure for the Oxirane Fragment Located at the End of Compound 15



Scheme 6. Coupling of Oxime-Probe 6 with Norbornene-Substituted Epoxomicin 16

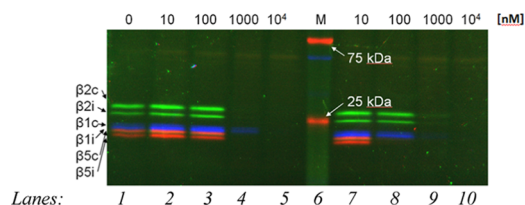
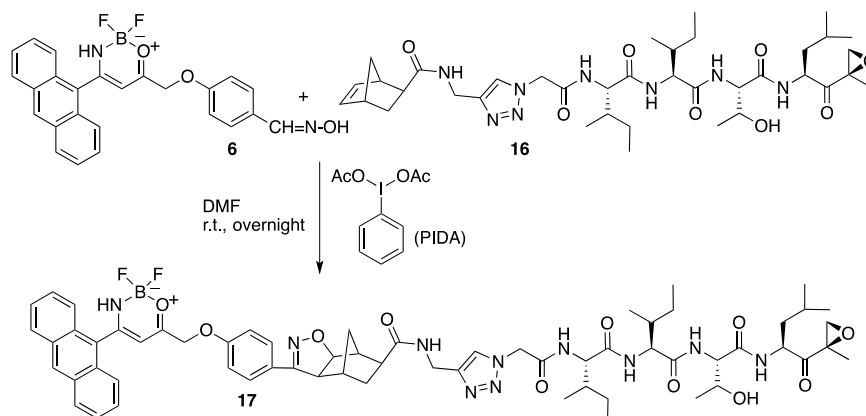


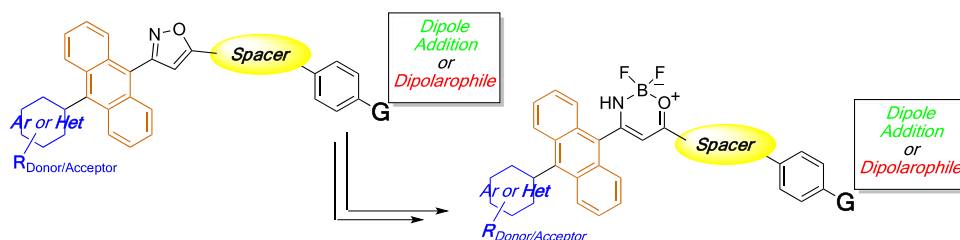
Figure 4. SDS-PAGE gel of compounds 15 and 17 (lanes 2, 3, 4, and 5, for increasing concentrations of probe 15; lanes 7, 8, 9, and 10 for increasing concentrations of probe 17; lane 1, specific fluorescent markers for β subunits; and lane 6, a molecular weight marker for reference).

fluorophores maintain their optical properties but the excitation was not efficient. Alternatively, the probes 15 and 17 were not strong enough from the fluorescence point of view to be detected with the instruments employed. We cannot exclude potential quenching or degradation (instability of the boron complex) during the interaction with the proteasome.

CONCLUSIONS

In conclusion, this work has shown the possible and reliable application of nitrile oxide chemistry in the biomedical field, especially as starting materials for the synthesis of some fluorescent probes. The chemistry of isoxazoles,²¹ dating to

many decades ago, in the field of imaging probes seems to be a promising and valuable alternative to azides.²² Azides, in fact, do suffer from the need of copper to perform correctly the cycloaddition. The use of copper and metals in general is detrimental in a biological environment, and the chemistry we offer by replacing azides with nitrile oxides is cleaner and safer; the construction of the boron complexes is simple, reliable, and robust. The methodology can be applied by taking advantage of the variety of structures having the aldehydes as starting compounds, to prepare corresponding nitrile oxides. In light of this, we have indeed demonstrated the possibility to expand the application of 1,3-dipolar cycloaddition reactions to obtain a group of easily derivatizable fluorescent probes. We were able to obtain two novel boron fluorescent probes 15 and 17 that enhance the range of reactions suitable for the coupling with proteasome inhibitors. For this purpose, the optical properties of these compounds were investigated and suggest the potential for employment as fluorescent tags in imaging techniques. The drop of fluorescence quantum yield values for compound 6 with respect to 5 remains a problem whose solution is not far from the synthetic route we have followed, while maintaining the validity of the chemical strategy. The moderate quantum yield values maintained in the DMSO/H₂O mixture confirm the suitability of these compounds in the cellular environment. The ABPP assays performed did not give

Scheme 7. Synthetic Strategy toward Newly Designed Fluorescent Probes^a

^aThe Spacer can be also deleted, leaving a fully conjugated system.

us the ultimate answer about the behavior of compounds **15** and **17** in SDS-PAGE analyses. The coupling tests performed showed good reactivity for the probes **15** and **17**, even with short peptide chains such as epoxomicin. Improvement of the fluorescence properties of enaminoketone-based boron complexes is needed and actively pursued. At present, the tuning of the optical properties of the probes is a crucial point for making these compounds competitive with commercial ABPP probes. The stability of the boron complex within the lysate environment is also a key point for its application in imaging techniques.

Design, synthesis, and application: these aspects must be synchronized and to do that, brand new isoxazole derivatives are currently under preparation by functionalizing the anthryl moiety with several aromatic rings bearing donor and acceptor substituents by means of the Suzuki coupling; moreover, selected heterocycles have been inserted too (Scheme 7).²³

This revised and enlarged library of isoxazoles was already verified to show unexpected intense fluorescent properties, even stronger than the corresponding boron complexes. Fluorescent studies are actively pursued to compare the photochemical behavior of the uncomplexed and complexed probe couples (see Scheme 7) to determine the real need of a boron complex to display optical properties in this type of compound. These findings will be communicated in a full paper that is under preparation.

EXPERIMENTAL SECTION

General Methods and Materials. Melting points (mp) are uncorrected. Elemental analyses were performed on a FlashSmart elemental analyzer. ¹H NMR and ¹³C NMR were registered on Bruker AVANCE 300 spectrometers in deuterated solvent solutions. The chemical shifts are expressed in ppm, using tetramethylsilane as the internal reference. IR spectra were registered using a FT-IR PerkinElmer RX-1 spectrometer dissolving the analyzed products in DCM (film).

Chromatographic columns were obtained using Kieselgel 60, 70–230 (Merck), with a BIOTAGE MPLC or BIOTAGE Isolera One with KP-SIL columns; the elution solvents were from cyclohexane/ethyl acetate, 9:1, to pure ethyl acetate. UV–vis spectra were registered with a Jasco V-550 UV–vis spectrophotometer; the fluorescence spectra were registered with a PerkinElmer LS 55 luminescence spectrometer.

Anthracenenitrile oxide **9** was prepared from the corresponding commercially available oxime according to the known procedure.^{24,3} Solvents and other reagents were purchased and used without any further purification.

Synthesis of 4-(Prop-2-yn-1-yloxy)benzaldehyde oxime (10). A solution of 3.20 g (46.0 mmol) of hydroxylamine hydrochloride in 20 mL of water was added to 4.83 g (30.1

mmol) of 4-(prop-2-ynoxy) benzaldehyde (**8**) dissolved in 125 mL of ethanol. NaOH solution was added to adjust the pH at 5, and the reaction was heated at 40 °C for 3 h. The mixture was then refluxed for 20 min and kept under stirring overnight. Ethanol was partially evaporated until the formation of a white solid, and water was added afterward. The mixture was extracted with diethyl ether, and the combined organic phases were dried over MgSO₄ and evaporated after filtration. The product was recrystallized from cyclohexane/ethyl acetate, obtaining a white solid of **10** in 87% yield (4.60 g). Mp: 68–70 °C from cyclohexane/ethyl acetate. FT-IR: $\nu_{C\equiv N}$ 1603 cm⁻¹; ν_{OH} 3262 cm⁻¹. ¹H NMR (δ , DMSO): 3.59 (s, 1H, C \equiv C–H); 4.83 (s, 2H, CH₂); 7.01 (AA'BB', 2H, arom.); 7.54 (AA'BB', 2H, arom.); 8.08 (s, 1H, N=C–H); 11.02 (s, 1H, OH). ¹³C NMR (δ , DMSO): 55.4; 78.4; 79.0; 115.1; 126.3; 127.7; 147.5; 158.0. Elemental analysis: calculated for C₁₀H₉NO₂ (MW = 175.06): C, 68.56; H, 5.18; N, 8.08; found: C, 68.55; H, 5.15; N, 8.09.

Synthesis of 4-((3-(Anthracen-9-yl)isoxazol-5-yl)methoxy)benzaldehyde Oxime (11). In a 250 mL round-bottom flask, 2.27 g (10.4 mmol) of anthracenenitrile oxide (**9**) was added dropwise to a solution of 1.89 g (10.8 mmol) of 4-(prop-2-ynoxy) benzaldehyde oxime (**10**) in 125 mL of anhydrous DCM. The reaction was kept in the dark and under stirring at room temperature for 48 h. Then, the mixture was diluted with DCM (100 mL) and the solution was washed with brine (3 × 50 mL) and dried over anhydrous Na₂SO₄.

The solvent was evaporated and the crude residue was purified by column chromatography, giving a yellowish solid of **11** in 66% yield (2.69 g). Mp: 128–131 °C from cyclohexane/ethyl acetate. FT-IR: $\nu_{C\equiv N}$ 1598 cm⁻¹; ν_{OH} 3265 cm⁻¹. ¹H NMR (δ , DMSO): 5.54 (s, 2H, CH₂); 7.04 (s, 1H, C=C–H); 7.19 (AA'BB', 2H, arom.); 7.58 (m, 6H, anthr.); 7.72 (m, 2H, anthr.) 8.14 (s, 1H, N=C–H); 8.20 (AA'BB', 2H, arom.); 8.83 (s, 1H, anthr.); 11.07 (s, 1H, OH).

¹³C NMR (δ , DMSO): 18.5; 60.7; 107.7; 115.3; 122.5; 124.9; 125.7; 126.7; 127.0; 127.9; 128.6; 129.0; 129.9; 130.7; 147.5; 158.4; 160.3; 168.4. Elemental analysis: calculated for C₂₅H₁₈N₂O₃ (MW = 394.13): C, 76.13; H, 4.60; N, 7.10; found: C, 76.15; H, 4.63; N, 7.09.

Synthesis of 4-((4-Amino-4-(anthracen-9-yl)-2-oxobut-3-en-1-yl)oxy)benzaldehyde Oxime (12). Compound **11** (1.80 mmol, 720 mg) was dissolved in 120 mL of a 9:1 acetonitrile/water mixture in a three-necked round-bottom flask. The solution was stirred under nitrogen for 15 min, and then 605 mg of molybdenum hexacarbonyl (2.30 mmol) was added. The reaction was stirred at 70 °C for 4 h.

DCM was then added, and the mixture was washed with brine (3 × 50 mL). The organic phase was dried over anhydrous Na₂SO₄ and evaporated. The crude product was purified on column chromatography, giving a beige-reddish

solid of **12** in 68% yield (491 mg). Mp: 183–189 °C from cyclohexane/ethyl acetate. FT-IR: $\nu_{\text{C}=\text{N}}$ 1579 cm^{-1} ; $\nu_{\text{OH/NH}_2}$ 3265 cm^{-1} . ^1H NMR (δ , DMSO): 4.66 (s, 2H, CH_2); 5.40 (s, 1H, $\text{C}=\text{C}-\text{H}$); 6.93 (AA'BB', 2H, arom.); [7.11] (AA'BB', 2H, arom.); 7.53 (AA'BB', 2H, arom.); 7.53 (m, 4H, anthr.); [7.85] (AA'BB', 2H, arom.); 7.97 (m, 2H, anthr.); 8.07 (s, 1H, $\text{N}=\text{C}-\text{H}$); 8.13 (m, 2H, anthr.); 8.47 (s, 1H, NH_2); 8.71 (s, 1H, ant.); 9.88 (s, 1H, OH); 10.25 (s, 1H, NH_2); [10.95] (s, 1H, OH). ^{13}C NMR (δ , DMSO): 70.8; 93.4; 93.5; 114.5; 114.8; 124.8; 125.2; 125.5; 126.2; 127.4; 127.5; 128.0; 129.5; 130.2; 130.7; 130.7; 131.3; 147.2; 158.5; 161.5; 161.7; 190.9; 191.2; 192.0. Elemental analysis: calculated for $\text{C}_{25}\text{H}_{20}\text{N}_2\text{O}_3$ (MW = 396.15): C, 75.74; H, 5.08; N, 7.07; found: C, 75.75; H, 5.03; N, 7.08.

Synthesis of 4-((4-(Anthracen-9-yl)-2,2-difluoro-2,3-dihydro-1 λ^3 ,3,2 λ^4 -oxazaborinin-6-yl)methoxy)benzaldehyde Oxime (6). Compound **12** (2.30 mmol, 916 mg) was dissolved in 100 mL of anhydrous DCM along with 10 mL of distilled triethylamine in a 250 mL three-necked bottom flask. The solution was stirred under a nitrogen atmosphere for 10 min, and then 4 mL of boron trifluoride etherate (32.4 mmol) was added. The reaction was stirred under a nitrogen atmosphere overnight. The mixture was then diluted with DCM (20 mL) and washed with brine (3 \times 50 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated, obtaining a dark oil. The crude product was then purified on column chromatography, giving a yellow solid of **6** in 40% yield (409 mg). Mp: 179–184 °C from from cyclohexane/ethyl acetate. FT-IR: ν_{NH} 3362 cm^{-1} ; ν_{OH} 3272 cm^{-1} ; $\nu_{\text{C}=\text{N}}$ 1614 cm^{-1} . ^1H NMR (δ , DMSO): 5.05 (s, 2H, CH_2); 6.06 (s, 1H, $\text{C}=\text{C}-\text{H}$); 7.03 (AA'BB', 2H, arom.); 7.51 (AA'BB', 2H, arom.); 7.62 (m, 4H, anthr.); 7.62 (bs, 1H, NH); 7.71 (m, 2H, anthr.); 8.07 (s, 1H, $\text{N}=\text{C}-\text{H}$); 8.23 (m, 2H, anthr.); 8.86 (s, 1H, anthr.); 11.03 (s, 1H, OH); [11.16] (s, 1H, OH). ^{13}C NMR (δ , DMSO): 30.7; 67.0; 97.6; 115.0; 123.8; 125.9; 126.5; 127.2; 127.7; 127.8; 128.8; 129.5; 130.4; 147.4; 158.1; 172.0; 175.2. Elemental analysis: calculated for $\text{C}_{25}\text{H}_{19}\text{BF}_2\text{N}_2\text{O}_3$ (MW = 444.15): C, 67.59; H, 4.31; N, 6.31; found: C, 67.55; H, 4.33; N, 6.30.

Synthesis of 3-((4-(Anthracen-9-yl)-2,2-difluoro-2,3-dihydro-1 λ^3 ,3,2 λ^4 -oxazaborinin-6-yl)methoxy)phenyl)-3a,4,5,6,7,7a-hexahydro-4,7-methanobenzo[d]isoxazole (13). PIFA (20 mg) was dissolved in 50 mL of DMSO along with boron complex **6** (123 mg). After 5 min, 150 mg of 2-norbornene was added and the mixture was stirred for 4 h. The mixture was then extracted with DCM, and the combined organic phases was dried over Na_2SO_4 . The solvent was evaporated and the product was purified by a chromatographic column, giving a yellow solid (**13**) in 81% yield (122 mg). Mp: >210 °C from ethyl acetate. FT-IR: $\nu_{\text{C}=\text{N}}$ 1598 cm^{-1} ; ν_{NH} 3361 cm^{-1} . ^1H NMR (δ , DMSO): 1.15 (d, 1H, norb.); 1.26 (d, 1H, norb.); 1.48 (m, 4H, norb.); 3.64 (d, $J = 8$, 1H, norb.); 4.55 (d, $J = 8$, 1H, norb.); 5.08 (s, 2H, CH_2); 6.06 (s, 1H, $\text{C}=\text{C}-\text{H}$); 7.07 (AA'BB', 2H, arom.); 7.63 (AA'BB', 2H, arom.); 7.63 (m, 4H, anthr.); 7.73 (m, 2H, anthr.); 8.85 (s, 1H, anthr.); 11.18 (s, 1H, NH). ^{13}C NMR (δ , DMSO): 22.1; 26.6; 31.8; 56.2; 67.0; 86.7; 97.6; 115.1; 122.3; 123.8; 125.8; 125.9; 127.2; 127.6; 128.2; 128.8; 129.5; 130.4; 130.7; 137.1; 156.0; 158.4; 172.0; 175.1. Elemental analysis: calculated for $\text{C}_{32}\text{H}_{27}\text{BF}_2\text{N}_2\text{O}_3$ (MW = 536.21): C, 71.66; H, 5.07; N, 5.22; found: C, 71.65; H, 5.03; N, 5.20.

Synthesis of (2S,3R)-2-(2-(4-(4-((4-(Anthracen-9-yl)-2,2-difluoro-2,3-dihydro-1 λ^3 ,3,2 λ^4 -oxazaborinin-6-yl)methoxy)phenyl)-1H-1,2,3-triazol-1-yl)acetamido)-N-(((2S,3R)-1-(((2S,3S)-3-hydroxy-1-(((S)-4-methyl-1-((R)-2-methylloxiran-2-yl)-1-oxopentan-2-yl)amino)-1-oxobutan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)-3-methylpentanamide (15). Alkynyl fluorescent probe **5** (2.71 mg, 6.37 μmol) was dissolved in 1 mL of argon-degassed DMF along with 3.64 mg (6.26 μmol) of azido-epoxomicin **14**. An aqueous solution of CuSO_4 (1 mg, 4 μmol) and sodium ascorbate (1 mg, 5 μmol) was added to the mixture, and the solution was then stirred overnight at room temperature under an argon atmosphere.

The crude product was purified by silica gel column chromatography (DCM/MeOH 9:1) to give 8.59 mg of cycloadduct **15** (6.37 μmol).

The product weight was initially higher than expected probably due to some water residue that was very difficult to remove. After a series of careful drying processes, the product, obtained in 55% yield, was submitted for relative characterization by mass spectrometry.

The mass spectrum (MS) reporting a mass value of 1023.30 m/z corresponds to the molecular weight of **15** (1006.94 m/z) plus OH (MW + OH).

Synthesis of (3aS,4R,6S,7S,7aS)-3-(4-((4-(Anthracen-9-yl)-2,2-difluoro-2,3-dihydro-1 λ^3 ,3,2 λ^4 -oxazaborinin-6-yl)-methoxy)phenyl)-N-(((1-((4S,7S,10S,13S)-4,7-di((R)-sec-butyl)-10-((S)-1-hydroxyethyl)-15-methyl-13-((R)-2-methyl-oxirane-2-carbonyl)-2,5,8,11-tetraoxo-3,6,9,12-tetraazahexadecyl)-1H-1,2,3-triazol-4-yl)methyl)-3a,4,5,6,7,7a-hexahydro-4,7-methanobenzo[d]isoxazole-6-carboxamide (17). To a solution of 1.59 mg (3.58 μmol) of oxime fluorescent probe **6** in argon-degassed DMF was added 1.74 mg (5.40 μmol) of PIDA. The solution was stirred for 10 min under an argon atmosphere, and then 1.98 mg (2.62 μmol) of norbornene-epoxomicin **16** was added to the solution. The mixture was stirred overnight at room temperature.

The crude product was purified by silica gel column chromatography (DCM/MeOH 9:1) to give 1.87 mg (1.56 μmol) of cycloadduct **17** (45% yield).

Due the known reactivity of nitrile oxides and the nature of the dipolarophile, the purification step was quite problematic and several steps of chromatographic purification were needed. The final cycloadduct **15** was obtained, and the MS spectrum confirmed its structure with MW = 1199.30 (theor. 1199.15) m/z .

Density Functional Theory (DFT) and Time-Dependent DFT Calculations. Calculations were performed using Gaussian 09, Revision E.01. All optimizations were performed using the SMD(DCM)-(TD)-CAM-B3LYP/6-31G(d,p)//(TD)-B3LYP/6-31G(d,p) level of theory (over the first 10 singlet states). The identity of the stationary points was confirmed by frequency analysis due to the absence imaginary frequencies.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00672.

Synthesis of 4-(prop-2-yn-1-yloxy)benzaldehyde (**8**); competitive ABPP assays (SDS-PAGE); UV-vis and fluorescent spectra of compounds **6**, **5**, **15**, and **17** in

DCM, MeOH, ethylene glycol, and DMSO/H₂O 1:1, as well as the corresponding fluorescent spectra and the quantum yield data; ¹H and ¹³C spectra of all new synthesized compounds; MS spectra of the final adducts **15** and **17** (PDF)

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Notes

The authors declare no competing financial interest.

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