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PAPER

Synthesis of an activated phosphonated bifunctional chelate with potential for PET imaging and radiotherapy†

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The synthesis of a phosphonated acyclic bifunctional chelate **L*** for the labeling of biomaterial is described. **L*** is based on a pyridine backbone, functionalized in *ortho* positions by aminomethyl-bis-methylphosphonic acids, and, in the *para* position, by a side chain containing a reactive NHS carbamate function. The stability of **L*** in aqueous solutions at different pH values was studied by mass spectrometry, showing the activated function to be sensitive to hydrolysis above neutral pH. The reactivity of **L*** towards amine functions was tested using ethylamine under different conditions of pH and concentrations, and by the labeling of two reference peptides containing both an N-terminal amino function and a ε-amino group of a lysine residue in the backbone, and a supplementary thiol group of a cysteine residue for one of these two peptides. The results showed the coupling to be efficient at pH 8.0, with a total selectivity for the terminal amine function with respect to lysine and cysteine. The labeling was further performed on B28-13, a mouse monoclonal antibody specifically recognizing tenascin-C protein in human cancer. The labeled antibody was characterized by means of mass spectrometry and spectrofluorimetry, unraveling a labeling ratio of one chelate per antibody. Finally, the affinity of the labeled antibody towards its target was controlled by immunofluorescence staining experiments on human colon cancer biopsies, confirming the affinity of the labeled peptide for tenascin-C.

Introduction

Owing to its favorable physical properties, ^{64}Cu ($T_{1/2}$ = 12.7 h; β^+: 0.656 MeV, 17.8%; β^-: 0.573 MeV, 38.4%)[1] is considered as a promising radionuclide for medicinal purposes in diagnostic imaging by positron emission tomography (PET) and as a surrogate to ^{67}Cu for radiotherapy.[2–6] For such applications, ^{64}Cu has to be delivered within a living organism as a stable Cu complex that is anchored to a biological target (protein, antibody, peptide or small molecule), which requires the development of bifunctional chelates (BFCs) characterized by the presence of a coordination site, able to rapidly and selectively form a stable complex, and of a chemically reactive function allowing conjugation to the target biomolecule.

So far, most stable chelates for Cu(II) complexation have been based on polyaza macrocyclic ligands such as cyclen (1,4,7,10-tetraazacyclododecane) and cyclam (1,4,8,11-tetraazacyclotetradecane) derivatives bearing coordinating acetate pendant groups like DOTA and TETA.[2–8] While these ligands appeared theoretically attractive for Cu(II) coordination regarding their thermodynamic stability constants, their application to PET imaging often remains difficult as a result of slow Cu(II) complexation kinetics[9] or *in vivo* instability for long term biomedical applications.[10,11]

In order to provide enhanced stability, several macrocyclic ligands incorporating methylenephosphonate pendant arms have been developed.[12–17] Indeed, replacement of carboxylic acid groups by phosphonic acid often resulted in an increased thermodynamic stability of the complexes, partly due to the higher electrostatic interactions of phosphonate groups compared to carboxylate ones.[18] While radiometal chelates bearing phosphonate functions have been described, some of them being grafted on peptides[12] or NHS-folate,[19] to our knowledge, no publication has been reported relating to the introduction of an activated chemical function that allows the coupling to biomolecules without the need for supplementary chemical coupling agents such as DCC or EDCI.

In our quest toward the development of BFCs for Cu(II), we recently communicated the synthesis of a novel acyclic ligand **L**

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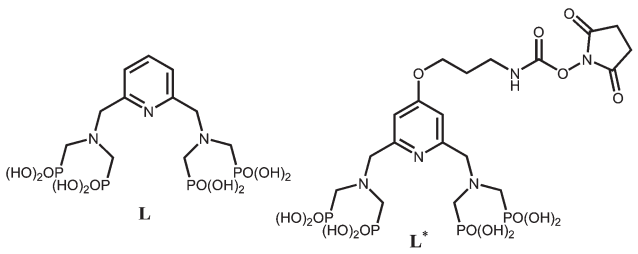


Scheme 1

based on a pyridine scaffold and featuring two bis(methanephosphonate)aminomethyl groups (Scheme 1).[20,21] At room temperature, **L** rapidly forms highly stable Cu(II) complexes (log K_{CuL} = 22.7) over a broad pH domain.

As expected, the introduction of phosphonated groups resulted in a large increase of the stability of the formed complexes relative to their carboxylated analogue ($\Delta\log K_{CuL}$ = 7.0).[20,22] **L** appeared to be a very efficient ligand for the complexation of Cu(II) at low concentrations of the metal and under physiological pH (pCu^{II} = 15.5 at pH = 7.4, $[L]_{tot} = 10^{-8}$ M and $[Cu]_{tot} = 10^{-9}$ M) and the complexation was shown to be very selective compared to those of other nearby transition metal cations such as Ni(II), Co(II), Zn(II) and also *vs*. Ga(III).[21] Considering the required properties for Cu PET radiotracers, such as high thermodynamic stability and kinetic inertness, fast complexation and selectivity for Cu(II), **L** can be considered as a very promising chelating ligand for ^{64}Cu, and may also find applications for the complexation of other hard cations such as lanthanides.[23]

These findings prompted us to elaborate a synthetic route to introduce a reactive function for further conjugation to targeted biomaterials. Herein, we disclose the preparation of a novel phosphonated bifunctional chelate **L*** derived from ligand **L** (Scheme 1) and containing a lateral chain with a reactive NHS carbamate group which is to our knowledge the first example of a phosphonated BFC activated with a reactive functional group. The stability of the BFC at various pH will be presented, together with labeling experiments with ethylamine and two reference peptides to define the optimum conditions for labeling. Finally, the labeling of B28-13, a mouse monoclonal antibody specifically recognizing tenascin-C protein in human cancer,[24] will be performed and characterized.

Results and discussion

Synthesis of L*

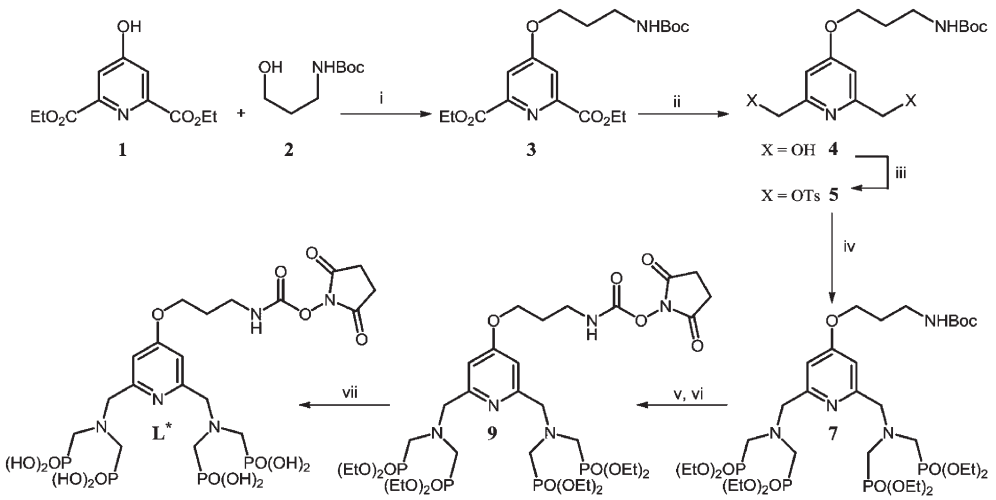
The synthetic strategy for the preparation of **L*** is depicted in Scheme 2. Diethyl chelidamate **1**[25] and *tert*-butyl (3-hydroxypropyl)carbamate **2**[26] reacted under Mitsunobu conditions to produce the key intermediate **3** in 83% yield. Reduction of the diester **3** with sodium borohydride in EtOH afforded the diol **4** (81%) which was converted in 85% yield into the bis-tosylated derivative **5** by treatment with a mixture of tosyl chloride and sodium hydroxide.[27] Under basic conditions, N-alkylation reaction between ditosylate **5** and tetra-ethyl iminobis(methanephosphonate) **6**[28] furnished the tetra-diethylester phosphonated intermediate **7** in 57% yield. Selective removal of the Boc protecting group was carried out quantitatively with TFA and the resulting trifluoroacetate salt **8** was converted into the NHS carbamate **9** by treatment with *N*,*N*′-disuccinimidyl carbonate and triethylamine in MeCN (65% for the two steps). Finally, a crucial step of the synthesis allowed the selective hydrolysis of phosphonic esters in the presence of the active NHS carbamate using an excess of TMSBr in the presence of 2,6-lutidine to provide, after methanolysis, the desired ligand **L*** as a lutidinium salt in 66% yield.

Stability of L* and coupling with amines

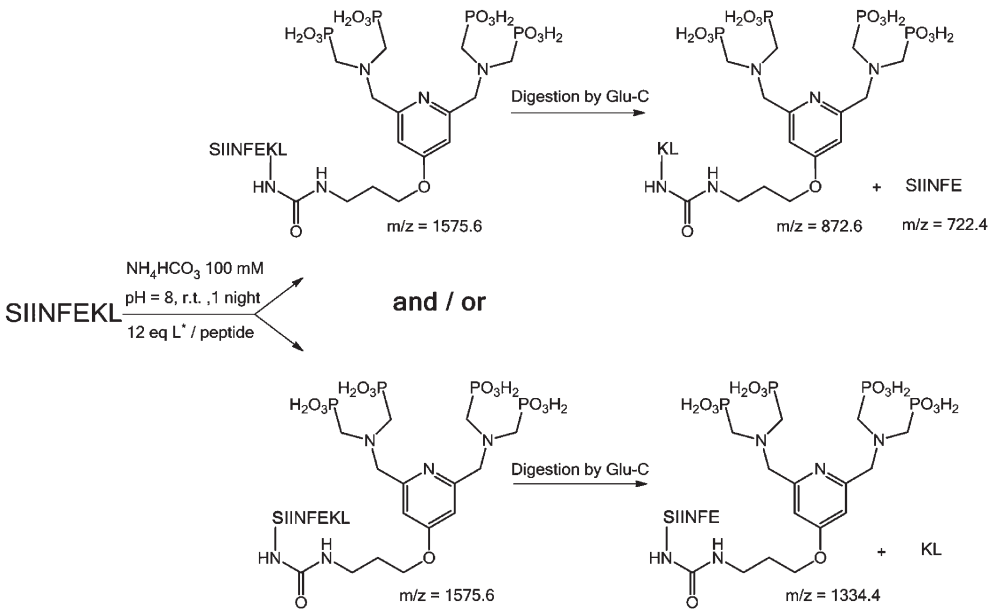
Stability of L* in aqueous solutions. Considering the reactivity of NHS esters and carbamate functions,[29,30] the stability of **L*** in aqueous solutions at different pH values was checked by electrospray ionisation mass spectrometry. In a typical experiment, **L*** was dissolved in an aqueous solution at fixed pH containing volatile buffers and its stability was monitored in time by following the intensity of the MS signals in the positive mode. At pH 4.0 (acidified with formic acid), the half-life of **L*** was measured to be 90 min, with a gradual loss in the peak of **L*** at 728 *m*/*z* units (Fig. S1, ESI†) and the appearance of a single new peak at 587 *m*/*z* units, attributed to the formation of the amine resulting from the hydrolysis of **L*** followed by decarboxylation of the formed carbamic acid (R–NH–C(O)OH). When the pH is raised to 6.8 (50 mM $AcONH_4$ buffer), the half-life was considerably shortened to 14 min and the decomposition products were observed to be a mixture of the previously observed free amine and of a second compound, with a peak at 630 *m*/*z* units, attributed to the carbamic acid. At pH 8.6 (50 mM $(NH_4)_2CO_3$), the hydrolysis is faster than the measuring conditions (less than one minute), essentially resulting in the saponification of the carbamate ester to the carbamic acid.

Coupling with amines. Regarding the fast kinetics for hydrolysis of **L*** at neutral and basic pH values, it was decided to verify first that the reactivity towards primary amine functions should be competitive for an efficient labeling. For that purpose, **L*** was incubated in solutions containing 40 equiv. of ethylamine at different pH values and the formation of the corresponding ethyl urea was monitored by ESI/MS by calculation of the intensity ratio *R* between the urea compound and the product of hydrolysis. Under these conditions, it was satisfactorily observed that *R* values of 0.80, 0.35 and 0.09 can be obtained at pH values of respectively 9.9, 9.5 and 8.6. Lower *R* values at low pH can be accounted for by the protonation of the amine at such pH (pK_{EtNH_2} = 10.7), but when the pH is raised, the formation of the urea is highly competitive with regard to hydrolysis. A very large excess of ethylamine at pH 9.9 did not bring significant improvements in the *R* value.

The coupling reaction was then studied with two model peptides: **S**IINFE**K**L and **K**LTPL**C**VSL. The first peptide presents two potential amine residues, the one of the terminal amine of the serine (denoted **S**) and the lateral *n*-butylamine of the lysine group (denoted **K**). The best conditions for coupling of **L*** to the peptide **S**IINFE**K**L were a pH of 8.0 and a ratio of 12 : 1 (**L***–peptide). The coupling product was observed at *m*/*z* 1575.5 (MH^+) in agreement with the calculated mass for a monolabeled peptide (*m*/*z* 1575.6). The labeled peptide was separated from the unlabeled one by reverse phase HPLC (Fig. S2, ESI†). The



Scheme 2 Synthetic protocol for the preparation of **L***. (i) DIAD, PPh_3, THF, 70 °C, 83%; (ii) $NaBH_4$, EtOH, 78 °C, 81%; (iii) TsCl, NaOH, THF/H_2O, rt, 85%; (iv) $HN(CH_2PO(OEt)_2)_2$ **6**, K_2CO_3, KI, MeCN, 70 °C, 57%; (v) CF_3CO_2H, CH_2Cl_2, rt, then (vi) DSC, NEt_3, MeCN, rt, 65% for the two steps; (vii) TMSBr, 2,6-lutidine, CH_2Cl_2, rt, then MeOH, 66%.



Scheme 3 Labeling scheme for peptide **S**IINFEK**L** and the products possibly obtained after digestion with Glu-C enzyme.

labeling site of coupling (terminal NH_2 or Lys ε-NH_2) was determined after digestion with *Staphylococcus aureus* protease V8 (Glu-C enzyme), which specifically cleaves a peptidic bond after glutamic acid residues (Scheme 3). In the mass spectrum, the observation of a peak at *m/z* 1334.4 corresponding to the labeled N-terminal fragment *SIINFE indicated that the coupling occurred on this site. No peak corresponding to the labeling of the lysine residue was observed, in agreement with the higher pK_a of Lys ε-NH_2 (pK = 10.8) *versus* terminal NH_2 of serine (pK = 9.2).

Finally, the complexation of Cu(II) by the labeled peptide was further confirmed by a fluorescence quenching experiment in which the fluorescence spectra of the labeled peptide were monitored as a function of the added Cu(II) (Fig. 1).

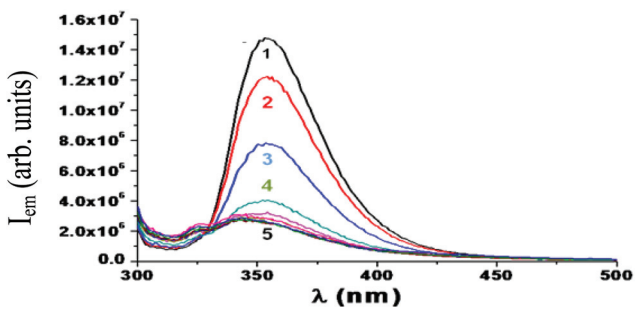


Fig. 1 Fluorescence titration of the labeled **S**IINFE**K**L peptide upon addition of 0 (black), 0.25 (red), 0.5 (blue), 0.75 (green) and excess (pink and black) equivalent of $CuCl_2$ (λ_{exc} = 270 nm).

In the absence of added Cu(II), excitation at 270 nm in the $\pi \rightarrow \pi^*$ transitions of the pyridyl moiety[31] of the BFC gave rise to a broad emission centered at around 355 nm. The addition of the Cu(II) salts resulted in a gradual quenching of this emission band, attributed to an intramolecular metal to ligand electron transfer process, as often observed with Cu(II).[32] Finally, the Cu complex was also detected by MALDI/MS spectrometry at *m/z* 1636.9 in agreement with the calculated mass (Fig. S3, ESI†).

The coupling of **L*** with the second model peptide was studied to check the selectivity of the reaction with an amine *versus* a thiol function. The cysteine containing peptide **K**LTPL**C**VSL was labeled by **L*** at pH 8.0 and the resulting peptide (*m/z* 1585.8, MH^+) was then treated with iodoacetamide, known to specifically react with cysteine residues. Mass spectrometry analysis revealed that the labeled peptide could be totally carbamidomethylated (*m/z* 1643.0, MH^+, Δ = 57 Da). This confirmed that the cysteine could still react with iodoacetamide and that it has not been labeled by **L***, as anticipated for an active NHS carbamate.

Antibody labeling

Labeling of B28-13. Tenascin-C (TNC) is a glycoprotein highly expressed during embryogenesis but largely restricted or absent in healthy adult tissues. Its expression is regained in several pathological situations[33] including many human cancer types.[34] Many antibodies against TNC have been developed, and some of them are currently used in preclinical or clinical trials.[35] The B28-13 antibody is a mouse monoclonal antibody specifically recognizing the C-terminal FN type III repeats 13–15 of human TNC,[24] a motif which is present in all TNC splice variants. B28-13 was thus chosen as a prototype antibody to be labeled by **L*** for potential future applications in tumor imaging by PET.

Characterization of B28-13 and its labeled version. Prior to labeling, a MALDI/MS analysis was carried out on the B28-13 antibody which was found to have a molecular mass of 148 000 Da (Fig. 2). The labeled and purified antibody was also analyzed by MALDI/MS. The mass spectrum shows a shift of the mono-charged peak $[M + H]^+$ to the higher masses due to the addition of the ligand **L***. At the maximum, this shift is of about 800 Da which would roughly correspond to one ligand per antibody.

Immunofluorescence staining. In order to verify that the labeling procedure has not perturbed the affinity of the antibody towards its antigen, immunofluorescence staining experiments were performed in which the affinities of B28-13 and BFC labeled B28-13 were checked using tissue sections of patient biopsies of human colorectal cancers (Fig. 3). The presence of the antibodies was revealed by counterstaining with an anti-mouse Cy3-labeled secondary antibody. Control experiments were also performed using biopsies of normal colon tissue and for the non-specific affinity of the secondary antibody. As evidenced in Fig. 3A and B, comparison of the labeled antibody and the non-modified B28-13 showed the specific TNC expression in the stroma of human colorectal cancer and at the basal lamina of normal human colon. This indicates intact tenascin-C antigen recognition after labeling with **L***.

Another series of immunofluorescent staining experiments were achieved on tissue sections of patient biopsies of human colorectal cancer to verify the affinity of the Cu(II) complex of the labeled antibody (Cu(II)-BFC labeled B28-13). Results also confirmed tenascin-C antigen recognition after labeling B28-13 with Cu(II)–**L*** (Fig. S4, ESI†).

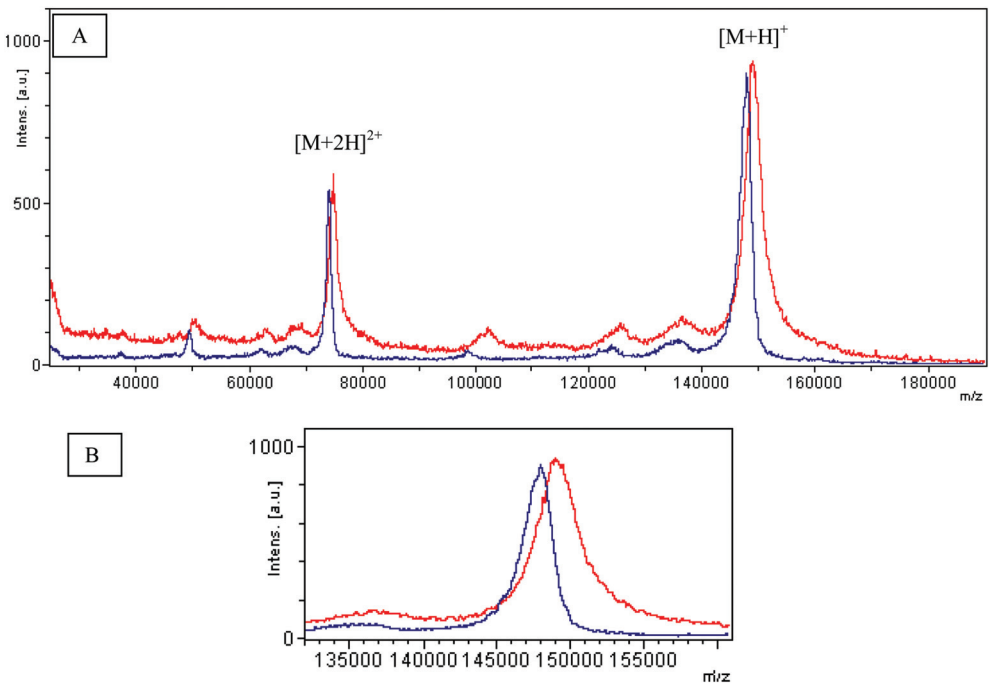


Fig. 2 (A) Superimposed MALDI/MS spectra of B28-13 (blue) and B28-13 labeled with **L*** (red). (B) Zoom on the *m/z* region from 135 000 to 160 000 showing the 800 units shift in mass due to the added chelate.

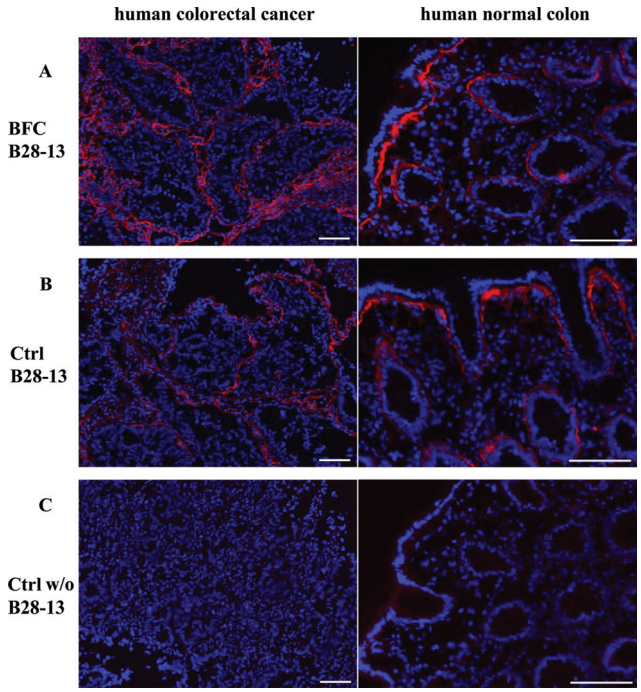


Fig. 3 Immunofluorescence staining on tissue sections of patient biopsies of human colorectal cancer (left column) or normal colon (right column) using BFC labeled B28-13 (A) or non-modified B28-13 (B) (scale bar = 100 μm, red = Cy3, blue = DAPI). Panel (C) shows the absence of a non-specific signal from the secondary antibody alone.

Conclusion

Following our previous findings on the efficient complexation of Cu(II) by ligand **L**, the present work demonstrates the feasibility to introduce a reactive *N*-hydroxysuccinimide carbamate function into the skeleton of the ligand **L**, providing a chemically activated bifunctional chelate **L*** ready for the labeling of biomolecules. Although **L*** is relatively sensitive to hydrolysis in aqueous solutions at neutral or basic pH, it can easily be conjugated in an aqueous solution to primary amines, small peptides and even large biomolecules such as the B28-13 antibody. As expected for such an activated function, the conjugation was selective toward terminal amino residues of the peptides and was not reactive towards thiol functions of cysteines. Labeling of B28-13 could be readily achieved under conventional conditions and the labeled antibody was characterized by mass spectrometry and spectrofluorimetry. Immunofluorescence staining experiments showed the labeled antibody to retain its affinity towards tenascin-C in human colorectal cancer.

In conclusion, we have developed the first example of an activated phosphonated bifunctional chelate and we demonstrated its ability to label biomolecules. Our efforts are now directed towards the use of such chelates for *in vivo* imaging applications with ^{64}Cu PET imaging, with a particular emphasis on the determination of the *in vivo* stability of the radiolabelled compound, but also for other potential metal based imaging techniques such as single photon emission computed tomography with ^{111}In or radiotherapeutic applications with the ^{67}Cu radioisotope.[36]

Experimental section

Synthesis of the compounds

General methods. Column chromatography and flash column chromatography were performed respectively on silica (0.063–0.200 mm, Merck) and silica gel (40–63 μm, Merck). Solvent mixtures used for TLC and flash column chromatography are reported in v/v ratios. ^{1}H and ^{13}C NMR spectra were recorded on an Avance 300 spectrometer at 300 MHz for proton frequency and 75 MHz for carbon frequency. ^{31}P NMR spectra (161.9 MHz) were recorded on an Avance 400 apparatus. Chemical shifts are given in parts per million, relative to the residual protic solvent peak.[37] IR spectra were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Scientific). Mass spectra (MS) of synthesized compounds and elemental analysis were obtained by the Service Commun d'Analyse de l'Université de Strasbourg.

THF was distilled over sodium and benzophenone under a nitrogen atmosphere. Other solvents were used as purchased. Triethylamine was distilled over potassium hydroxide prior to use and other commercially available reagents were used without further purification.

Diethyl chelidamate **1**,[25] *tert*-butyl (3-hydroxypropyl)carbamate **2**[26] and tetra-ethyliminobis-(methanephosphonate) **6**[28] were synthesized according to the literature procedures.

Diethyl 4-(3-[(*tert*-butoxycarbonyl)amino]propoxy)pyridine-2,6-dicarboxylate 3. To a solution of diethyl chelidamate **1** (3.27 g, 13.70 mmol) in THF (325 mL) were added under nitrogen, triphenylphosphine (7.20 g, 27.46 mmol) and *tert*-butyl (3-hydroxypropyl)carbamate **2** (4.80 g, 27.52 mmol) diluted in THF. DIAD (5.43 mL, 27.4 mmol) was added dropwise and the mixture was stirred at 70 °C for 12 h before being cooled to rt. The solvent was removed under reduced pressure and the oily residue was purified by two flash chromatographies (respectively with petroleum ether/EtOAc 70/30 and with CH_2Cl_2/MeOH 100/0 to 98.5/1.5) yielding ether **3** (4.5 g, 83%) as an off-white solid. R_f = 0.63 (CH_2Cl_2/MeOH 96/4). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.44 (s, 9H), 1.45 (t, J = 7.1 Hz, 6H), 2.05 (qt, J = 6.3 Hz, 2H), 3.34 (q, J = 6.3 Hz, 2H), 4.20 (t, J = 12.6 Hz, 2H), 4.47 (q, J = 7.1 Hz, 4H), 4.81 (s broad, 1H), 7.74 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.2, 28.4, 29.4, 37.5, 62.4, 66.6, 79.4, 114.3, 150.2, 156.0, 164.7, 166.8. Found: C, 57.35; H, 7.02; N, 6.93. Calc. for $C_{19}H_{28}N_2O_7$: C, 57.56; H, 7.12; N, 7.07%. ESI$^+$/MS: m/z 397.2 ([**3** + H]$^+$, 100%). IR (cm^{-1}, ATR): ν 3411, 3250, 2980, 1737, 1724, 1703, 1509, 1338, 1252, 1238, 1165, 1107, 1029, 784.

***tert*-Butyl (3-[(2,6-bis(hydroxymethyl)pyridin-4-yl)oxy]propyl)-carbamate 4.** To diester **3** (4.2 g, 10.5 mmol) in ethanol (210 mL) was added in portions $NaBH_4$ (2.0 g, 52.92 mmol). The mixture was refluxed for 2 h before being cooled to rt. Water was added and then solvents were removed *in vacuo*. The residue was partitioned between water and CH_2Cl_2 and the aqueous layer was extracted with CH_2Cl_2 (4 × 50 mL). The combined organic layers were washed with a saturated aqueous solution of NH_4Cl followed by water, dried with Na_2SO_4, filtered and concentrated under vacuum. The resultant white solid **4** was collected and used without purification (2.66 g, 81%). R_f = 0.36 (CH_2Cl_2/MeOH 90/10). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.44

(s, 9H), 2.01 (qt, J = 6.3 Hz, 2H), 3.33 (q, J = 6.3 Hz, 2H), 4.09 (t, J = 6.0 Hz, 2H), 4.71 (s, 4H), 6.71 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.5, 29.4, 37.6, 64.4, 65.7, 79.4, 105.6, 156.3, 161.2, 166.5. Found: C, 56.10; H, 7.72; N, 8.80. Calc. for $C_{15}H_{24}N_2O_5$: C, 57.68; H, 7.74; N, 8.97%. ESI$^+$/MS: m/z 313.2 ([**4** + H]$^+$, 100%), 355.2 (33%). IR (cm^{-1}, ATR): ν 3372, 2974, 1685, 1603, 1573, 1520, 1273, 1146, 1049, 1009, 844.

(4-(3[(*tert*-Butoxycarbonyl)amino]propoxy)pyridine-2,6-diyl)-bis(methylene)bis(4-methylbenzenesulfonate) 5. A solution of TsCl (3.55 g, 18.64 mmol) in THF (67 mL) was added dropwise to a 0 °C cooled mixture of diol **4** (1.45 g, 4.66 mmol) and NaOH (1.12 g, 27.96 mmol) in THF/H_2O (1:1, 34 mL). The mixture was warmed to rt and stirred for 5–6 h. The two layers were decanted and the aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with a 5% aqueous solution of $NaHCO_3$ followed by brine, dried with Na_2SO_4, filtered and evaporated under vacuum. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 99/1 to 98/2) to afford bis-tosylated compound **5** (2.44 g), as a white solid, in 85% yield. R_f = 0.28 (CH_2Cl_2/MeOH 98.5/1.5). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.46 (s, 9H), 1.99 (qt, J = 8.3 Hz, 2H), 2.44 (s, 6H), 3.31 (q, J = 6.6 Hz, 2H), 4.04 (t, J = 6.1 Hz, 2H), 4.70 (s broad, 1H), 4.98 (s, 4H), 6.81 (s, 2H), 7.34 (d, J = 8.1 Hz, 4H), 7.81 (d, J = 8.3 Hz, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.8, 28.5, 29.4, 37.6, 66.1, 71.3, 79.6, 107.7, 128.2, 130.1, 132.8, 145.3, 155.3, 156.1, 166.7. Found: C, 55.49; H, 5.94; N, 4.36. Calc. for $C_{29}H_{36}N_2O_9S_2$: C, 56.11; H, 5.85; N, 4.51%. ESI$^+$/MS: m/z 643.2 ([**5** + Na]$^+$, 100%). IR (cm^{-1}, ATR): ν 3357, 2926, 1677, 1367, 1172, 1034, 955, 840, 810, 670.

***tert*-Butyl (3-[(2,6-bis[(bis[(diethoxyphosphoryl)methyl]amino)-methyl]pyridin-4-yl)oxy]propyl)carbamate 7.** To a solution of tetra-ethyliminobis(methanephosphonate) **6** (350.2 mg, 1.10 mmol) in MeCN (22 mL), under a nitrogen atmosphere, freshly *flame-dried* K_2CO_3 (400 mg, 2.88 mmol) was added. The mixture was stirred for 20 min and compound **5** (300.0 mg, 0.48 mmol) in MeCN (50 mL) and freshly *flame-dried* KI (167.0 mg, 1.00 mmol) were added. The reaction mixture was heated at 70 °C for 40 h. After filtration, the solvent was removed *in vacuo* and the crude oil was purified by flash chromatography (CH_2Cl_2/MeOH 100/0 to 90/10). Compound **7** was obtained as an oily light yellow product (249.0 mg, 57%). R_f = 0.33 (CH_2Cl_2/MeOH 92/8). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.23 (t, J = 6.4 Hz, 24H), 1.35 (s, 9H), 1.90 (m, 2H), 3.17 (m, 10H), 4.03 (m, 22H), 5.09 (s broad, 1H), 6.99 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.4 (d, J = 6.2 Hz), 28.4, 29.2, 37.5, 50.3 (dd, J = 158 Hz, J = 8.6 Hz), 62.0 (d, J = 6.4 Hz), 62.4 (t, J = 8.2 Hz), 65.5, 79.0, 108.3, 156.0, 159.4, 166.3. ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 24.60. Found: C, 45.64; H, 7.92; N, 6.15. Calc. for $C_{35}H_{70}N_4O_{15}P_4$: C, 46.15; H, 7.75; N, 6.15. ESI$^+$/MS: m/z 933.4 ([**7** + Na]$^+$, 100%). IR (cm^{-1}, ATR): ν 3303, 2980, 1709, 1597, 1250, 1231, 1160, 1019, 959, 777.

2,5-Dioxopyrrolidin-1-yl-(3-[(2,6-bis[(bis[(diethoxyphosphoryl)-methyl]amino)methyl]pyridin-4-yl)oxy]propyl)carbamate 9. To a solution of amine **7** (1.21 g, 1.33 mmol) in 15 mL of dichloromethane was added dropwise at 0 °C, under nitrogen, TFA (1.97 mL, 26.6 mmol). The mixture was stirred at rt for 9 h. The solvent was evaporated to dryness. The crude trifluoroacetate salt **8** was dried under vacuum to give an oil which was used directly in the next step. R_f = 0.10 (CH_2Cl_2/MeOH 88/12). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31 (t, J = 7.1 Hz, 24H), 2.29 (m, 2H), 3.30 (m, 10H), 4.13 (m, 16H), 4.32 (s broad, 4H), 4.46 (m, 2H), 7.39 (s, 2H), 7.92 (s broad, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.2 (m), 26.1, 37.1, 50.4 (dd, J = 158.9 Hz, J = 6.1 Hz), 57.6 (t, J = 5.8 Hz), 63.5 (t, J = 3.4 Hz), 67.7, 110.7, 115.5 (q, J = 289.0 Hz, CF_3CO_2H), 155.7, 160.2 (q, J = 39.7 Hz, CF_3CO_2H), 171.8. ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 24.05. ESI$^+$/MS: m/z 406.2 ([**8** + 2H]$^{2+}$, 100%), 811.3 ([**8** + H]$^+$, 32%). IR (cm^{-1}, ATR): ν 2989, 1774, 1634, 1202, 1160, 1023, 975, 797, 704.

To a solution of salt **8** (1.33 mmol) in MeCN (30 mL) under nitrogen, triethylamine (0.33 mL, 2.39 mmol) and DSC (612.2 mg, 2.39 mmol) in MeCN were added. The reaction mixture was stirred at rt for 5 h 45. The solvent was evaporated to dryness and the residue dissolved in dichloromethane. The organic layer was washed with a saturated aqueous solution of NH_4Cl until TLC analysis showed complete disappearance of the DSC residue. The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure to yield NHS carbamate **9** as a pale brown oil (821.0 mg, 65% for the two steps). R_f = 0.60 (CH_2Cl_2/MeOH 88/12). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.27 (t, J = 7.1 Hz, 24H), 2.02 (m, 2H), 2.76 (s, 4H), 3.17 (d, J = 9.8 Hz, 8H), 3.36 (m, 2H), 4.09 (m, 20H), 4.27 (m, 2H), 7.25 (s broad, 2H), 7.57 (s broad, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.5 (m), 25.5, 28.5, 38.7, 50.6 (dd, J = 160.0 Hz, J = 8.0 Hz), 61.4, 62.2 (m), 65.8, 108.9, 152.0, 158.9, 169.9. ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 24.13. ESI$^+$/MS: m/z 869.3 (77%), 952.3 ([**9** + H]$^+$, 100%). IR (cm^{-1}, ATR): ν 3224, 2983, 1780, 1737, 1599, 1208, 1096, 1019, 961, 776, 731.

({[(4-(3-({[(2,5-Dioxopyrrolidin-1-yl)oxy]carbonyl}amino)-propoxy)pyridine-2,6-diyl)bis(methylene)]bis(azanetriyl)}tetra-*kis*(methylene))tetraphosphonic acid L*. To a solution of NHS carbamate **9** (51.0 mg, 0.053 mmol) in CH_2Cl_2 (3 mL), under nitrogen, 2,6-lutidine (0.32 mL, 2.75 mmol) then TMSBr (0.27 mL, 2.04 mmol) were added and the resulting mixture was stirred at room temperature for 15 h. The solvents were evaporated. The crude was coevaporated with CH_2Cl_2 then methanol (5 mL) was added to the residue and was evaporated under reduced pressure. The last procedure was realized twice again. The resulting solid was centrifugated in CH_2Cl_2 and washed several times with CH_2Cl_2 and methanol to afford final ligand **L*** as a pale brown lutidinium salt (36.0 mg, 66%). ^{1}H NMR (D_2O, 300 MHz): δ 2.08 (m, 2H), 2.72 (s, 6H, *lutidine*), 2.91 (s, 4H), 3.45 (m, 2H), 3.63 (d, J = 12.1 Hz, 8H), 4.27 (m, 2H), 4.91 (s, 4H), 7.13 (s, 2H), 7.63 (d, J = 8.1 Hz, 2H, *lutidine*), 8.28 (t, J = 8.0 Hz, 1H, *lutidine*). ^{13}C NMR (D_2O, 100 MHz): δ 18.7 (*lutidine*), 25.3, 27.6, 38.2, 52.4 (d, J = 139.5 Hz), 59.0, 66.6, 110.8, 124.6 (*lutidine*), 146.0 (*lutidine*), 151.2 (*lutidine*), 153.0, 153.3, 167.9, 173.9. ^{31}P NMR (D_2O, 161.9 MHz): δ 7.88. Found: C, 30.50; H, 4.61; N 8.32. Calc. for $C_{19}H_{33}N_5O_{17}P_4 \cdot C_7H_9N \cdot 2HBr \cdot H_2O$: C, 30.79; H, 4.57; N, 8.28. ESI$^+$/MS (H_2O/MeCN/formic acid): m/z 728.1 ([**L*** + H]$^+$, 100%). IR (cm^{-1}, ATR): ν 3203, 2959, 1773, 1730, 1627, 1609, 1206, 1156, 1047, 920, 797.

Labeling of model peptides and B28-13 antibody

Stability of L* in aqueous solutions. Ligand solutions were extemporaneously prepared at different pH values: pH = 4.0 (adjusted with formic acid); pH = 6.8 (50 mM $AcONH_4$) and pH = 8.6 (50 mM $(NH_4)_2CO_3$) and infused directly into the ESI/MS instrument (MicrOTOF-Q, Bruker Daltonics). The infusion flow was 180 μL h^{-1} and the spectra were acquired over 1 min across the *m*/*z* range of 50–1500. The mass spectrometer was calibrated using an ESI-L Low Concentration Tuning Mix solution (Agilent Technologies). The intensities of peaks at 587, 630 and 728 *m*/*z* units were monitored at different times.

Labeling of the SIINFEKL model peptide. Mentioned quantity for the ligand **L*** has been calculated taking into account the estimated proportion (by ^{1}H NMR) of **L*** alone (25%) in the crude luditinium salt mixture. 500 μL of a 100 mM NH_4HCO_3 solution, 20.5 μL of a 10 μg μL^{-1} solution of the **S**IINFE**K**L peptide (0.21 μmol, 1 equiv.) and 1.18 mg of ligand **L*** (1.62 μmol, 7.75 equiv.) were successively added in an eppendorf. The pH of the medium was adjusted to 8 with a 100 mM NH_4HCO_3 solution and the reaction mixture was left overnight at room temperature.

MALDI/MS analysis of the labeled SIINFEKL peptide. 10 μL of the reaction medium were acidified by a 10% (v/v) TFA solution. 0.6 μL was then spotted on a polished steel MTP 384 target plate (Bruker Daltonics) with 0.6 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution using the dried droplet method. The mass spectrometer (Ultraflex, Bruker Daltonics) was operated in positive reflectron mode.

Purification of the labeled SIINFEKL peptide. The reaction medium was purified by RP-HPLC using a Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 μm). The labeled peptide was eluted at 14.5 min (flow rate 0.8 mL min^{-1}) using a gradient of 10 to 45% of solvent B over 30 min (solvent A: 0.1% (v/v) TFA in ultrapure water, solvent B: 0.1% (v/v) TFA in MeCN).

Digestion of the labeled SIINFEKL peptide by Glu-C enzyme. 0.5 μg of endoproteinase Glu-C (*Staphylococcus aureus* Protease V8) were added to 5 μg of the purified labeled **S**IINFE**K**L peptide in a 100 mM NH_4HCO_3 solution (pH = 7.8) and the reaction medium was left for 3 h at room temperature. After acidification using a 10% TFA solution, the medium was analyzed by MALDI/MS using THAP (2,4,6-trihydroxyacetophenone) as a matrix (10 mg mL^{-1} in 50% MeCN/50 mg mL^{-1} ammonium citrate solution in a ratio of 9 : 1 (v/v)). A control digestion experiment was performed on a native **S**IINFE**K**L peptide.

Fluorescence study of the labeled SIINFEKL peptide. Fluorimetric titration was performed on 400 μL (5×10^{-5} M) of the purified labeled **S**IINFE**K**L peptide. The measurements were performed on a Horiba Jobin Yvon spectrofluorimeter. The emission was measured at the maximum λ = 355 nm and the excitation wavelength was λ = 270 nm. The emission value was recorded for the labeled product first then by each successive addition of a 2.5×10^{-4} M Cu(II) solution (0.25 equiv. per addition).

Labeling of the KLTPLCVSL model peptide. Mentioned quantity for the ligand **L*** has been calculated taking into account the estimated proportion (by ^{1}H NMR) of **L*** alone (25%) in the crude luditinium salt mixture. 17 μL of a 100 mM NH_4HCO_3 solution, 3 μL of a 10 μg μL^{-1} solution of **K**LTPL**C**VSL (30 nmol, 1 equiv.) and 0.16 mg of ligand **L*** (0.22 μmol, 7.45 equiv.) were successively added in an eppendorf. The pH of the reaction medium was adjusted to 8 with a 100 mM solution of NH_4HCO_3 and the reaction mixture was left overnight at room temperature.

MALDI/MS analysis of the labeled KLTPLCVSL peptide. 10 μL of the reaction medium were acidified by a 10% (v/v) TFA solution. 0.6 μL of the reaction medium was then spotted on the target with 0.6 μL of the THAP matrix solution as described before.

Cysteine alkylation. 5 μL of the **K**LTPL**C**VSL labeling medium (3 μg of the peptide, 3 nmol, 1 equiv.), 5 μL of a 50 mM $(NH_4)_2CO_3$ solution (pH = 8.6) and 8 μL of a 1 μg μL^{-1} solution of iodoacetamide (40 nmol, 13 equiv.) were added in an eppendorf. The reaction medium was left for 1 h at room temperature in the dark, then acidified by a 10% (v/v) TFA solution and analyzed by MALDI/MS with THAP as a matrix.

Preparation of B28-13 antibody. The B28-13 antibody was obtained by affinity purification using a 1 mL HiTrap Protein G HP column (17-0404-01, GE Healthcare) connected to the AKTAPrime™ purification system (GE Healthcare). 100 mL of the supernatant collected from hybridoma cell cultures secreting B28-13[24] were used and purification performed according to the manufacturer's instructions. The obtained antibody eluate was dialyzed against PBS and stored in aliquots at −20 °C. SDS-PAGE followed by Coomassie staining was performed to verify purity of the antibody preparation.

MALDI/MS analysis of the antibody B28-13. Prior to analysis, both native and labeled antibody solutions were desalted on a C18 ZipTip (Millipore). 70 pmol of the antibody was loaded and then eluted in 5 μL H_2O/MeCN/formic acid 20/75/5 (v/v/v). 0.6 μL of the eluate was then spotted on the target plate with 0.6 μL of sinapinic acid (2 mg mL^{-1} in 50% MeCN). The mass spectrometer (Autoflex, Bruker Daltonics) was operated in positive linear mode, with BSA as a calibrant.

Labeling of B28-13. Mentioned quantity for the ligand **L*** has been calculated taking into account the estimated proportion (by ^{1}H NMR) of **L*** alone (26%) in the crude luditinium salt mixture. The labeling experiment was carried out on 350 μg (500 μL of a 0.7 mg mL^{-1} solution in PBS, 1.55 nmol) of the antibody B28-13 to which 0.22 mg of ligand **L*** (0.3 μmol) was added, corresponding to 200 equiv. of the ligand for each antibody (a higher ligand/protein ratio was used in the labeling of B28-13 to take into account the higher number of lysine residues present in an antibody and to be sure of having a good labeling ratio; this means that the reaction medium needed to be purified to eliminate the excess of **L***). The reaction medium was adjusted to a pH of 7.1 with PBS buffer and was stirred for 1 h at room temperature. The reaction medium was purified repeatedly to eliminate the excess of ligand with 10 mM Tris-HCl

pH 7.4 using Vivaspin500 (Sartorius) modules with a 30 kDa cut-off (500 µL of Tris-HCl per cycle; 20 cycles).

Immunostaining experiment. Histological analysis was performed by immunofluorescence staining on 7 µm sections of fresh frozen tissue biopsies of human colorectal cancer or normal colon. For experiments to verify affinity of BFC labeled B28-13, tissue sections were incubated for 45 min with 5% normal goat serum (NGS)/PBS and coupled or non-coupled B28-13 antibody preparations were diluted 1 : 50 in 5% NGS/PBS. For experiments to verify affinity of Cu(II)-BFC labeled B28-13, tissue sections were incubated for 45 min with 5% normal donkey serum (NDS)/PBS and coupled, copper coupled and non-coupled B28-13 antibody preparations were diluted in 5% NGS/PBS in the ratios of 1 : 800, 1 : 400 and 1 : 400 respectively. Then sections were incubated in a humidified chamber at 4 °C overnight followed by a Cy3-labeled anti-mouse secondary antibody (1 : 1600; 715-165-150, Jackson ImmunoResearch) for 1.5 h at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and microscopic analysis performed on a Zeiss Imager.Z2 microscope. Tissue samples had been acquired upon written consent according to conventional ethical standards.

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