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A new isoluminol reagent for chemiluminescence labeling of proteins

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ABSTRACT

We report the synthesis and the characterization of *N*-(4-aminobutyl)-*N*-ethyl-isoluminol (ABEI) macrocyclic lactone, an activated ester with an unusual macrocyclic structure, and its use for efficient ABEI conjugation to proteins. Compared to the equivalent reagent normally used for chemiluminescence protein labeling, the ABEI macrocyclic lactone displays improved chemical properties, including stability and reactivity. We show the simple synthesis and the use of ABEI macrocyclic lactone for efficient chemiluminescence labeling of monoclonal antibody mixtures currently used in clinical immunodiagnostic assays for the detection of the HIV p24 antigen in patients.

Chemiluminescent methods have become established in both routine clinical analysis and for clinical research applications.[1] Historically, luminol and isoluminol were the first chemiluminescent compounds to be used as labels to be conjugated to reporter molecules but they were surpassed in some applications by the more sensitive acridinium esters.[2] However, proteins, peptides, and antibodies conjugated to isoluminol are successfully employed in a significant number of commercially available in vitro immunoassays. Very recently, luminol and isoluminol have been employed in electroluminescence (ECL) biosensors[3] for immunoassays,[4] DNA assays,[5] as well as to develop probes for rapid bacterial detection,[6] and test kits for analysis of medical and food samples. *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI), is of relatively higher chemiluminescence efficiency compared to luminol and is the label of choice for more recent applications.[7] The chemical method commonly employed for the conjugation of ABEI to proteins or other biomolecules is based on the well established reactivity of *N*-hydroxysuccinimide (NHS) active esters of ABEI-succinate or -glutarate.[8] NHS esters react in aqueous media with the amino groups of lysines on protein surface thus forming amide conjugates. NHS esters suffer however of limited stability: at 0 °C at pH 7, their half-life is typically 4–5 h,[9] at pH 8.6 and 4 °C it drops to only 10 min.[10] Despite their instability to hydrolysis, NHS esters of luminol and isoluminol derivatives, in particular the ABEI-succinate or -glutarate NHS are currently the reagents of choice for protein conjugation.

Here we describe the synthesis of ABEI lactone **1**[11] with a macrocyclic structure. This compound has been easily obtained by a one-step procedure from the precursor ABEI-glutarate **2** (Scheme 1). ABEI macrocyclic lactone **1** readily reacts with protein lysines affording conjugation product **4** with an amide bond connecting ABEI to protein identical to that obtained by using the ABEI-glutarate NHS active ester reagent **3** (Scheme 1). ABEI macrocyclic lactone **1** was synthesized starting from commercial ABEI, that was reacted with glutaric anhydride in anhydrous pyridine (at 50 °C for about 20 min) to obtain the ABEI-glutarate **2** (quantitative yield after column purification) that was then treated with diisopropylcarbodiimide (DIC) in DMF at rt for 48–120 h obtaining ABEI macrocyclic lactone **1** in 40% yield with purity major to 98% after column purification.

The structure of compound **1** has been determined on the basis of mono- and bi-dimensional NMR (Supplementary data) and X-ray analysis. ^{1}H NMR spectrum in *d*-DMSO shows a sharp signal at 12 ppm corresponding to proton linked to O1, that exchanges with deuterated water. The presence of this proton strongly suggests that the phthalhydrazide moiety of **1** is in the lactim (cyclic enamide) form when dissolved in *d*-DMSO (Scheme 1). The

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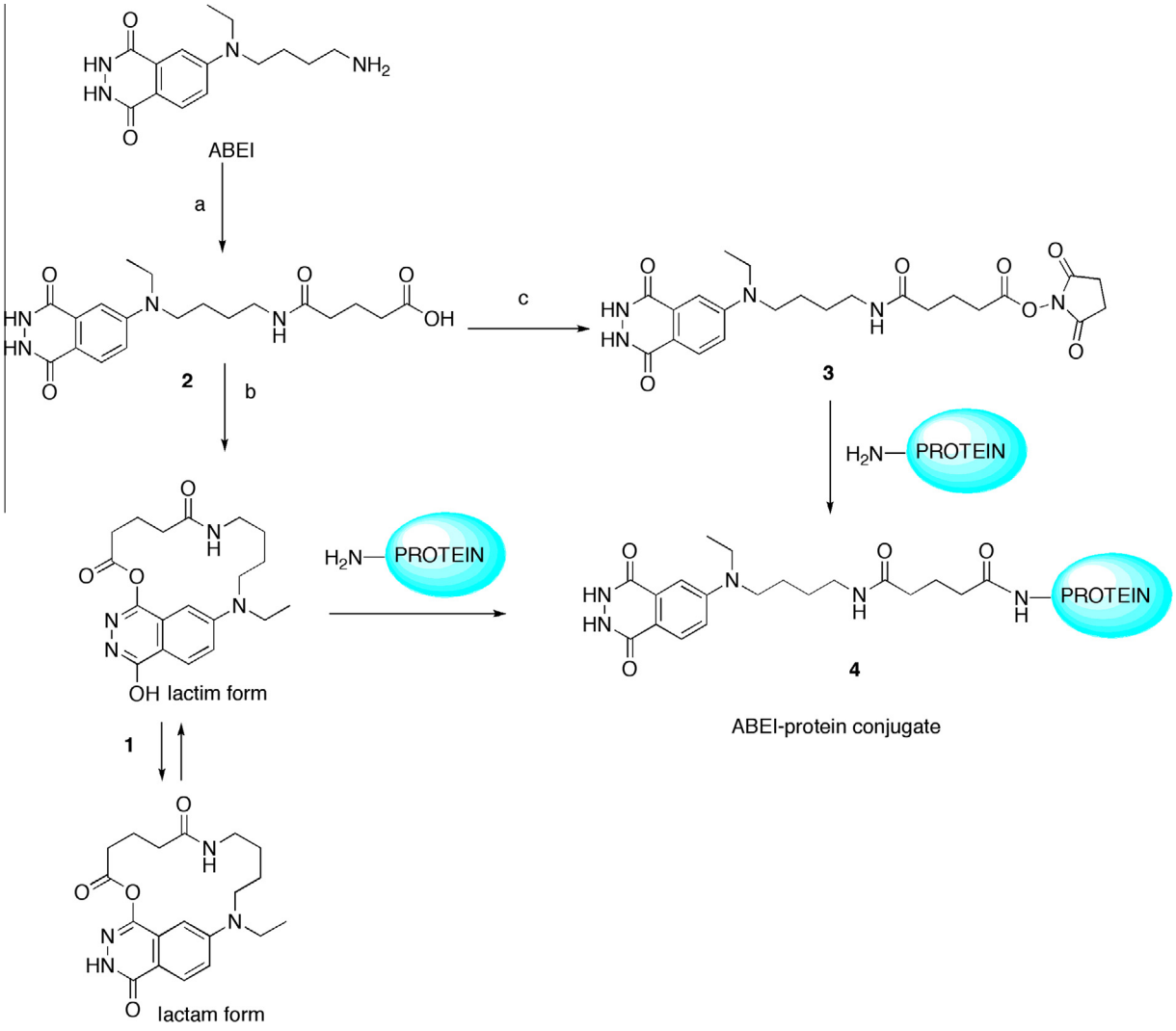

Scheme 1. Synthesis of ABEI-macrolactone **1** and conjugation with target proteins. Reagents and conditions: (a) glutaric anhydride, Py dry, 50 °C, 20 min, quant. yield; (b) DIC, DMF dry, rt 48–120 h, 40%; (c) NHS, DIC, DMF dry, 120–144 h, 95%.

presence of the lactim tautomer in solution is also confirmed by the comparison of ESI-mass spectra obtained from solutions of **1** in acid or basic aqueous media (9:1 water/DMSO with, respectively, 0.1% formic acid and 0.1% Et_3N). The spectrum of **1** in acid solution clearly shows the protonated lactim (MH^+ 373.1 Da) while the spectrum of **1** in basic solution shows the deprotonated form (negative ion mode, M371.3 Da, Supplementary data). 2D-NOESY experiments, performed at different mixing times at 35 °C, show three sets of interactions between the proton linked to N4, C16, and C12; C7, C9, and C10 and C5; C11, C12, and C18 (Supplementary data) that suggest a macrocyclic structure, also confirmed by X-ray analysis. The structure of the ABEI macrocyclic lactone **1**, as determined by X-ray diffraction, is illustrated in Figure 1.

In the phthalhydrazide moiety of **1,** at variance with the results in eliminate the solution reported above, the overall geometry is consistent with the lactam arrangement of the N2–C2–O1 group. In particular, the C2–O1 bond distance [1.236(2) Å] is appropriate for a C=O bond, not for a phenolic C–O bond (about 1.35 Å).[12] In the ester moiety, the C1–O4 bond [1.391(2) Å] is about 0.05 Å shorter than the corresponding one in alkyl esters. Conversely, the C19–O4 bond [1.373(3) Å] is 0.03 Å longer than in aliphatic esters.[13] The ester plane is significantly tilted with respect to the isoluminol moiety, the angle between normals to their average planes being 69.0 °, a common observation for aromatic esters. These findings, taken together, support the view that a lone pair on the O4 atom is involved to a significant extent in a phenol-type conjugation with the isoluminol aromatic system that occurs at the expense of the conjugation with the ester carbonyl group, thus well accounting for the 'active ester' properties of this derivative.[14] In the macrocycle connecting the O4 atom to the N3 atom, the amide bond between the N4 and C15 atoms is in the *trans* disposition. Also, most of the torsion angles about the C–C bonds are close to the *trans* disposition, except for those at the level of the C14 and C17 atoms (g^+ and g^-, respectively). As a result, the macrocycle is roughly square in shape, with the C14 and C17 atoms at the corner positions. The (carbonyl ester) O3 and the (carbonyl amide) O2 atoms protrude in opposite directions with respect to the average plane of the macrocycle.

It is known that ABEI enamide oxygens (O4 and O1) react as nucleophiles with activated acyl groups in intermolecular reactions. The carbodiimide-promoted intramolecular reaction observed in this case is however unprecedented. Except for the old example of DCC-promoted lactonization reaction employed by Woodward for reserpine synthesis[15] carbodiimide reagents, often combined with DMAP have been used rarely in macrolactonizations. This is mostly because of the formation of an unreactive *N*-acyl urea byproduct by rearrangement of the activated diimide through *N*-acyl migration (compound **6**, Scheme 2). As an example,