A new isoluminol reagent for chemiluminescence labeling of proteins

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Chemiluminescent methods have become established in both routine clinical analysis and for clinical research applications. 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. 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 eclectroluminescence (ECL) biosensors for immunoassays, DNA assays, as well as to develop probes for rapid bacterial detection and test kits for analysis of medical and food samples. 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.

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A B S T R A C T

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.

ABEI macrocyclic lactone 

The structure of compound 1 has been determined on the basis of mono- and bi-dimensional NMR (Supplementary data) and X-ray analysis. 1H 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
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% Et₃N). 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, M⁻ 371.3 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 macrocyclic 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 synthesis¹⁵ 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,
the major product in the macrolactonization of 15-hydroxypentadecanoic acid is the N-acyl urea byproduct, and the hexadecanolide is only isolated in 4% yield.\(^{16}\) In the case of ABEI-glutarate, the intramolecular reaction of the urea adduct 5 with O4 is probably faster than acyl migration, thus giving 1 in satisfactory yield (Scheme 2).

The stability of 1 dissolved in organic solvents (DMSO) was evaluated at two different storage temperatures, \(-30^\circ C\) and \(+4^\circ C\), by monitoring the formation of the hydrolysis product (ABEI-glutarate 2) by HPLC. Less than 2% of 1 was hydrolyzed to 2 after 385 days at \(-30^\circ C\), while less than 8% was hydrolyzed at \(+4^\circ C\) after 52 days (Supplementary data). 50% of 1 is hydrolyzed after 5 h at \(25^\circ C\) and at pH 8. These data clearly point out a greater stability of ABEI macrolactone 1 compared to the corresponding ABEI-glutarate NHS ester 3.

The labeling efficacy of ABEI macrocylic lactone 1 versus the corresponding NHS ester 3 was then verified by conjugating these compounds to two different anti-HIV p24 monoclonal antibodies.

**Figure 1.** X-ray diffraction structure of 1 with atom numbering. Displacement ellipsoids are drawn at the 30% probability level.

**Scheme 2.** Mechanism of carbodiimide-promoted macrolactonization. The acyl-urea byproduct 6 is often observed in DIC/DCC-promoted macrolactonizations, while in the case of ABEI adduct 5 the attack of O-4 favors macrolactonization.\(^{16}\)
(mAb) used as chemiluminescent tracers in the LIAISON® XL HIV antibody/antigen (Ab/Ag) assay (Fig. 2). This assay uses a mix of different anti-p24 mAbs in order to detect a wide array of HIV virus subtypes. The recognition of the HIV p24 protein (antigen, Ag) allows to detect the acute phase of HIV infection before patient’s antibody response thus reducing the serological window (time between the beginning of the infection and the day of virus detection) from 12 to 2–3 weeks. A high sensitivity is required for this test in order to detect concentrations of about 30 pg/mL of HIV antigen present in serum samples of the patient. Anti-p24 monoclonal Abs BMP24 and IGP24 were reacted with 1 and 3 under the same experimental conditions with the exception of the molar excess of the reagent used. In particular, 3 was employed in the same molar excess (46×) currently used for the industrial preparation of all antibody reagents used in the LIAISON® assay, while 1 was used at half of this concentration. The resulting Ab–ABEI conjugates were purified by size-exclusion chromatography and the relative degree of ABEI incorporation into proteins was expressed by the ratio of the absorbances at 280 nm (main protein contribution) and 329 nm (main ABEI contribution) (Supplementary data). This ratio (Fh) is therefore inversely proportional to the amount of ABEI incorporated into the protein. Despite 1 being used in half molar excess than 3, the incorporation efficiency measured as Fh was greater for 1 than for 3 for both antibodies (Table 1). Different molar excesses were used for the two reagents in order to obtain comparable values of Fh in the final conjugates.

A comparative immunometric activity of ABEI-labeled antibodies was then assessed by the sandwich chemiluminescence immunoassay (CLIA) for the qualitative detection of the HIV viral antigen p24. This test is based on the formation of an Ab–Ag–Ab immunocomplex (sandwich) deriving from the specific interaction of p24 HIV antigen with biotinylated and ABEI-labeled anti-p24 mAb (Fig. 2).

A serum (or plasma) sample is incubated for 10 min with a mixture of two biotinylated anti-p24 mAb, and subsequently with a mixture of anti-p24 mAb labeled with ABEI (tracers). The resulting immunocomplexes are then captured by streptavidin-coated magnetic particles in a 20-min incubation. The unbound material is removed by an appropriate wash buffer after a magnetic separation of magnetic microparticles, then the chemiluminescent reaction is induced and the luminescence detected. BPM and IGP anti-p24 antibodies labeled with 1 or 3 were used as tracers in the sandwich assay.

Serial dilutions of HIV viral lysate diluted in a pool of HIV negative samples were used as positive samples where the 1:4 million dilution represents the cut-off, that is the minimum concentration of HIV p24 Ag that can be distinguished from a negative sample with acceptable specificity and reproducibility. Seven different samples from blood donors were used as negative samples (Supplementary data). The mean signal of the seven negative samples was considered the background of the system. The ratio of the mean signal generated by positive samples and the background of the system (mean signal of the negative samples) was determined for the different tracer mixtures prepared with 1 or 3 as tracers in the sandwich assay. As a result of the higher number of ABEI molecules loaded, the tracer mixture prepared using 1, compared to those prepared with 3,
gave higher and more reproducible signal-background ratios (Fig. 3a) and increased assay sensitivity, as indicated by the increased signals obtained at the cut-off level (Fig. 3b). These results indicate that the use of ABEI macrolactone 1 allows to obtain protein tracers giving increased immunoassay sensitivities compared to reagent 3.

In conclusion, a new isoluminol-based reagent for protein labeling has been described, consisting in the macrolactone structure 1 derived from the ABEI glutarate 2. Compared to the ABEI N-hydroxysuccinimide ester, the ABEI-macrolactone shows a remarkably higher stability toward the hydrolysis, which translates into an improved labeling efficacy. This property, together with the easy and efficacious synthetic route, makes the ABEI macrocyclic lactone a better labeling agent for the preparation of chemiluminescent reagents for immunodiagnostic and research applications.

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Supplementary data

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References and notes