Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3595–3598

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

TRACERMER™ SIGNAL GENERATORS: AN ARBORESCENT APPROACH TO THE INCORPORATION OF MULTIPLE CHEMILUMINESCENT LABELS

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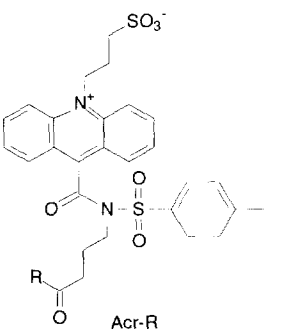
Received 24 August 1998; accepted 28 October 1998

Abstract: The synthesis, conjugation, and chemiluminescent evaluation of zero, first, and second order acridinium-based Tracermer™ signal generators are described. Members of this family of labels have potential use as *tracers* in diagnostic assays and are structurally similar to arborol dendri*mers*. Tracermer™-BSA conjugates showed up to a sixfold increase in light emission compared to the normal acridinium label.

Assays based on chemiluminescence are exquisitely sensitive with detection limits of attomoles (10^{-18} mol) and below.[1,2] While this is already impressive, detection of even lower levels of an analyte may be desirable. For some clinical markers detection of a single molecule would be ideal. Several approaches to extending the detection limits of chemiluminescent assays might be considered. One is to design a chemiluminophore with a higher quantum yield. However, after many years of research, the efficiency shown by the acridinium salts that are now widely used in chemiluminescent assays has not been surpassed. A second means to increase the sensitivity of the assay might be to simply increase the number of chemiluminophores on the bioconjugate. In this case, the label may modify a sufficient number of functional groups on the bioconjugate to the extent that it no longer has the solubility or specificity needed to perform optimally in the assay. This drawback might be overcome by incorporating more chemiluminophores per label (i.e., using a scaffold loaded with chemiluminophores instead of directly labeling the material). We decided to concentrate our efforts on this approach using our recently reported acridinium salt (**1**)[3] as the chemiluminophore and arborol dendrimers[4] based on monomer **2**. We have given the name Tracermer™ signal generators to this class of conjugates since they have potential use as *tracers* in diagnostic assays and have an arborol dendri*mer*ic core.



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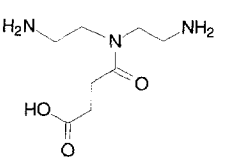

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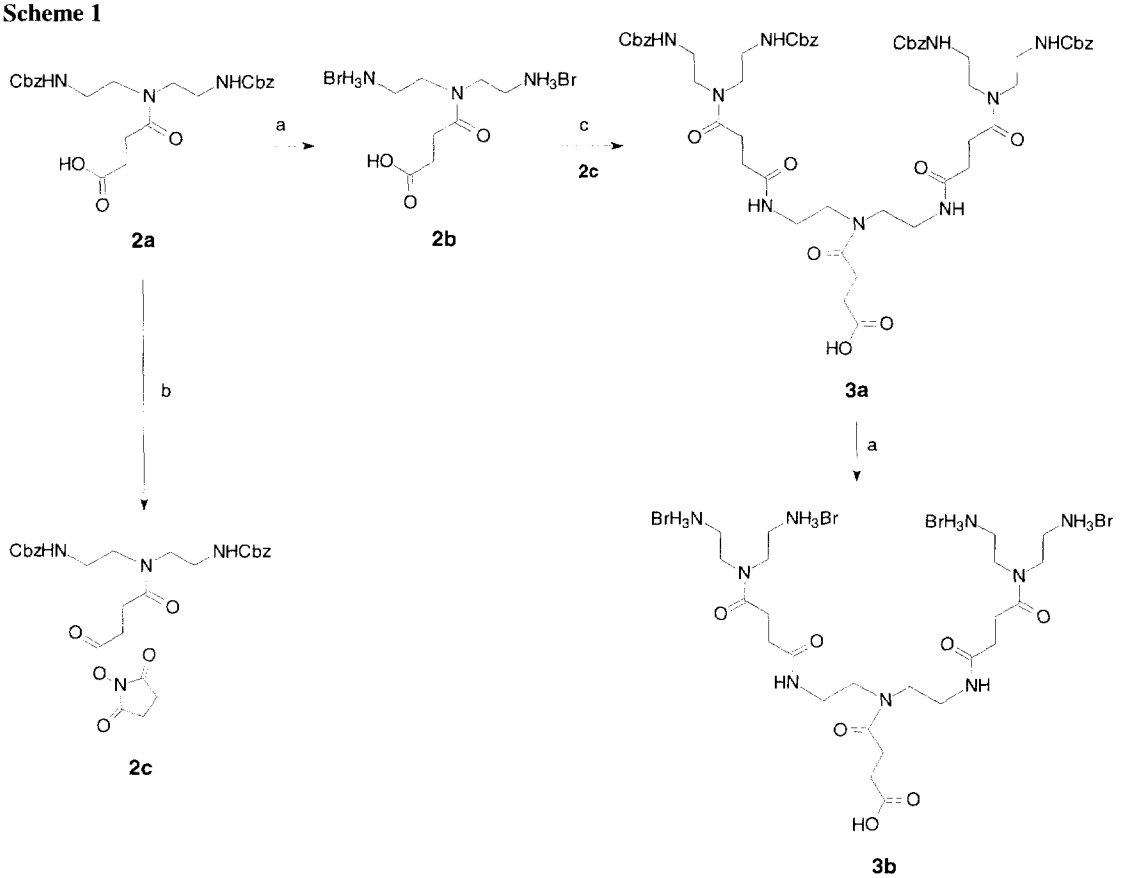
PII: S0960-894X(98)00656-8

The arborol scaffolds were prepared as shown in Scheme 1 starting from the previously reported Cbz-protected diamine **2a**.[5] Deprotection with HBr in acetic acid led to the first generation Tracermer™ backbone **2b** in 96% yield. The second generation Tracermer™ framework proceeded from **2a** via activation of the free carboxylic acid group with EDAC/NHS to give the active ester **2c**. Coupling with diamine **2b** in DMF/ buffer afforded **3a.** Removal of the Cbz protecting groups gave the tetraamine **3b** in an overall yield of 47% from **2b**.

Scheme 1

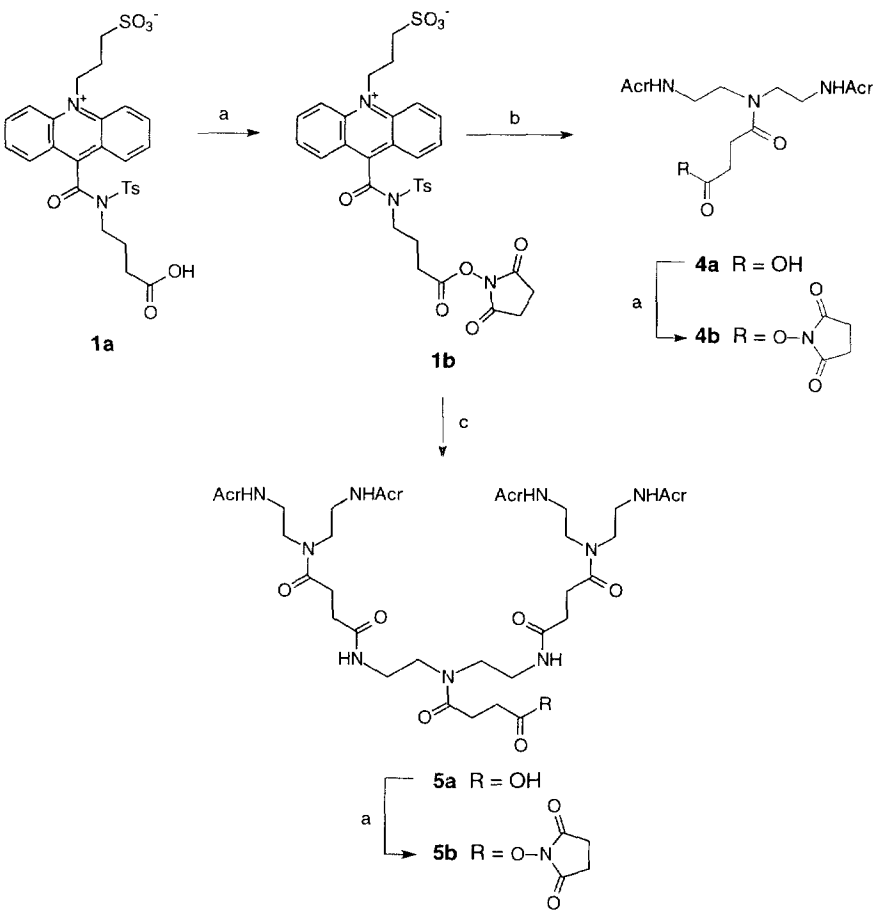


(a) 30% HBr/AcOH, CH_2Cl_2, 4 h; (b) 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide hydrochloride, *N*-hydroxysuccinimide, DMF, 14 h; (c) DMF, phosphate buffer, pH 7.8, 18 h.

The active esters of Tracermer™ signal generators were prepared from the requisite scaffold as shown in Scheme 2. The parent acridinium chemiluminophore **1a** was activated using *N*-succinimidyl trifluoroacetate (NHS-TFA)[6] to give the acridinium-NHS ester **1b** in 95% yield and greater than 97% purity without any chromatographic purification. Reaction of this active ester with diamine **2b** gave the free acid **4a** in 74% yield after preparative reversed-phase HPLC. Subsequent activation with NHS-TFA, gave the first generation Tracermer active ester **4b**. Preparation of the active ester of second generation Tracermer™ **5b** followed the same scheme.

To test the efficacy of the new labels, conjugates with bovine serum albumin (BSA) were prepared by acylating the protein with Tracermers **4b** and **5b**, and for comparison, acridinium-NHS ester **1b** in DMF/buffer (pH 8). The level of label incorporation determined by differential UV spectroscopy was 0.5–2.0 moles of label per mole of protein.[7]

Scheme 2



(a) *N*-succinimidyl trifluoroacetate, pyridine, DMF, 20 h; (b) **2b,** DMF, phosphate buffer, pH 7.8, 18 h; (c) **3b,** DMF, phosphate buffer, pH 7.8, 18 h.

The chemiluminescent emission (in counts/mol/label) of each Tracermer™-BSA conjugate was compared to that of the conjugate with the parent acridinium compound **1a.** The average counts per mole of label for the conjugates **1-BSA**, **4-BSA**, and **5-BSA** were determined to be $0.85 \pm 0.1 \times 10^{20}$, $2.0 \pm 0.2 \times 10^{20}$, and $5.3 \pm 0.7 \times 10^{20}$, respectively. Thus, the observed sixfold increase in light emission validates the

Tracermer™ approach to improving the chemiluminescence potential of conjugates. Future efforts will be directed towards the synthesis and evaluation of higher orders of Tracermer™ signal generators and their application in the development of various assays. Such efforts will be reported in due course.

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7. Experimental: The Tracermer™ conjugates were purified first by dialysis against aq NH_4OAc (10 mM), then by gel permeation chromatography [0.1% CHAPS/50 mM sodium phosphate, pH 6.3) The ratio of Tracermer™ label to BSA was determined by differential UV spectroscopy using the following formula:

$$r = \frac{A_{369}/\varepsilon_{369}}{A_{280}-(A_{369}/x)/45000\ M^{-1}\ cm^{-1}}$$

Where A_{280} and A_{369} are the absorbance readings of the BSA conjugate, x is the A_{369}/A_{280} ratio of the unconjugated Tracermer™ label, ε_{369} is the extinction coefficient of the Tracermer™ label at 369 nm, and 45000 M^{-1} cm^{-1} is the ε_{280} value for BSA.[8]

Chemiluminescent Analysis: The concentration of chemiluminescent label in each purified Tracermer™-BSA conjugate solution was determined using the appropriate extinction coefficient for **1a** ($\varepsilon_{369\ (20\%\ DMF/H_2O)}$ = 14000 $M^{-1}cm^{-1}$), **4a** ($\varepsilon_{369\ (20\%\ DMF/H_2O)}$ = 32000 $M^{-1}cm^{-1}$), and **5a** ($\varepsilon_{369\ (20\%\ DMF/H_2O)}$ = 54000 $M^{-1}cm^{-1}$). Chemiluminescence from the conjugate was triggered on a Berthold luminometer using a solution of sodium peroxide (0.18 N NaOH, 0.7% H_2O_2, 1% Triton X-100). Each BSA conjugate solution was serially diluted in DMF/H_2O (11% v/v) such that the emitted light was within the linear range of instrument detection. Light emission was expressed as the total relative light units in counts integrated over 30 seconds following the injection of the trigger solution. Counts/mol of Tracermer™ label were determined from the slope of the linear plot of relative light units in counts versus moles.
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