Synthesis of Highly Fluorescent Dienophiles for Detecting Conjugated Dienes in Biological Fluid

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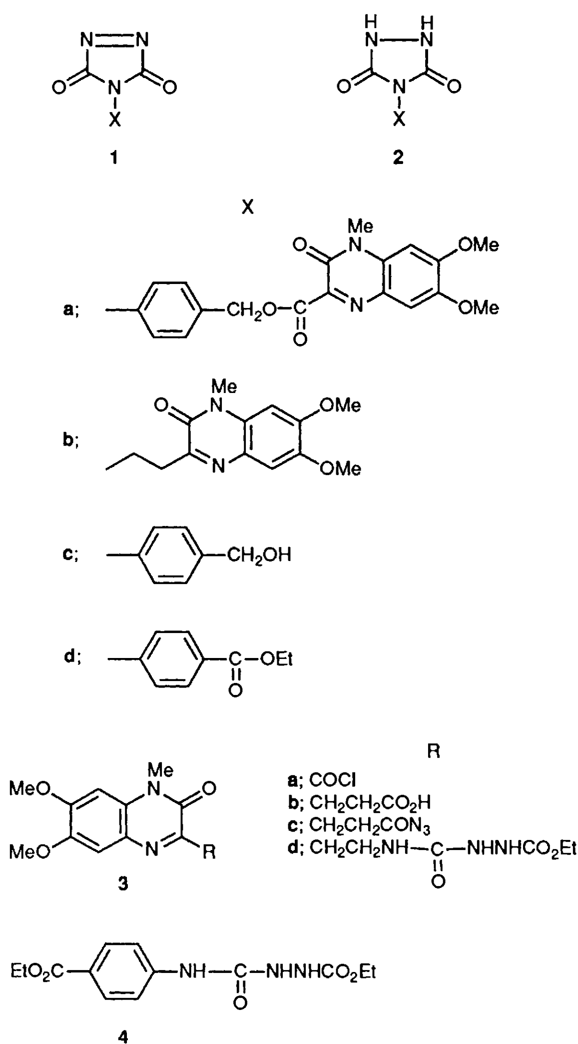
Fluorigenic dienophiles, 1,2,4-triazoline-3,5-dione derivatives with a highly fluorescent substituent at the 4-position **1a, b,** were synthesized as reagents for detecting biological compounds with a conjugated diene group.

Fluorescence labelling is one of the most sensitive methods for detecting very small amounts of a compound. To use the method to assay trace amounts of biological compounds in serum or tissues, the labelling reagent must react with the substrates with extremely high efficiency and with high substrate selectivity, as well as having high fluorescent efficiency. Because most of the known fluorescence-labelling reagents target common functional groups[1] such as amino, hydroxy, and carboxy groups, they lack specificity and the conditions for derivatization are rather too vigorous to apply to sensitive compounds. We have designed highly fluorescent and highly reactive dienophiles, **1a**, **b**, as reagents to detect biological compounds having a conjugated diene group, such as vitamin D metabolites, provitamin D, and vitamin A metabolites, with high selectivity. We now report the first synthesis of highly fluorescent dienophiles **1a**, **b**.

4-Substituted 1,2,4-triazoline-3,5-dione[2] was chosen as the dienophilic group because it is one of the most reactive dienophiles known, and has C_2 symmetry so it yields adducts as a single regioisomer. As a fluorigenic group, 6,7-dimethoxyquinoxalinone was selected because of its high fluorescent efficiency ($\phi_F \sim 0.99$)[3] and its stability against oxidation.

We synthesized the target compounds **1a**, **b** by the following two methods. The dienophile **1a** was synthesized by combining the nucleophilic triazoline synthon **2c** and the electrophilic fluorigenic synthon **3a** followed by oxidation. Ethyl 4-isocyanatobenzoate was combined with ethyl carbazate and the resulting semicarbazide **4** was cyclized under basic conditions (K_2CO_3, EtOH, reflux) to give the triazolidine **2d**,† the ester group of which was selectively reduced by diisobutylaluminium hydride (THF, −20 °C) to give **2c** in 62% overall yield from the isocyanate. The coupling of **2c** with the quinoxalinone carbonylchloride **3a**[3,4] was rather difficult. Under the usual conditions in the presence of pyridine, either the NH–NH or the OH group of **2c** reacted with **3a** to give a complex mixture of *N*- and *N*,*O*-substituted products. The desired ester **2a** was obtained in good yield (65%) by adding an amount of **2c** in DMF to a boiling solution of **3a** in benzene. The triazolidine **2a** was oxidized with iodobenzene diacetate[5] (DMF, room temperature, 30 min, quantitative) to give an

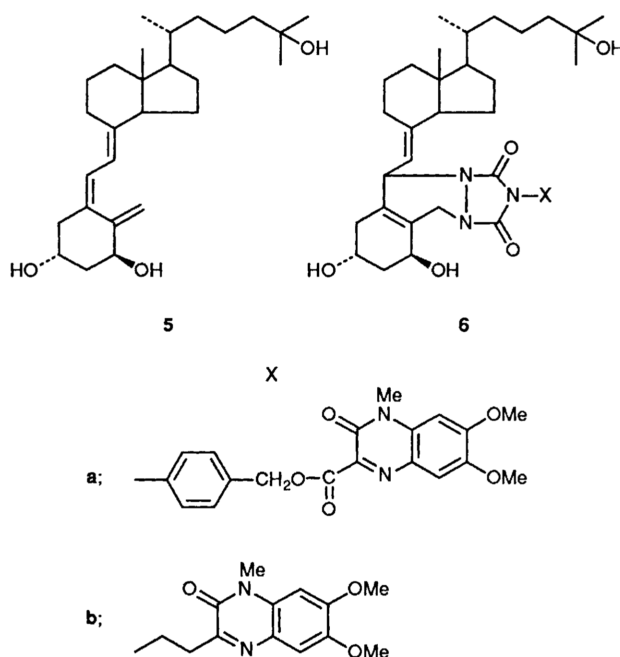
† Satisfactory analytical and spectral (^{1}H NMR, IR, UV, mass) data were obtained for all new isolable compounds.



orange–red solution of **1a**.‡ The adducts **6a** of 1α,25-dihydroxyvitamin D_3 **5**, the active vitamin D_3 metabolite, with **1a** was synthesized in nearly quantitative yield by adding a solution of **1a** to a solution of **5** at room temperature.

The dienophile **1b**, in which the dienophilic and fluorigenic groups are connected with a stable C–C bond, was obtained as a stable crystalline solid. The compound **1b** was synthesized from quinoxalinone 2-propionic acid **3b**, which was obtained from 2,7-diaminoveratrol in two steps in 63% overall yield. The carboxy group of **3b** was converted to a triazolidine ring *via* the Curtius rearrangement of the acyl azide **3c**. The conversion of the carboxylic acid **3b** to the acyl azide **3c** was successful only when diphenylphosphoryl azide (DPPA)[6] was used. Thus, **3b** was treated with DPPA (Et_3N, DMF) and the acyl azide **3c** obtained was transformed *in situ* to the corresponding isocyanate (reflux, benzene, 1 h) which was then treated with ethyl carbazate (reflux, benzene, 30 min) to give the semicarbazide **3d** in 65% overall yield. The semicarbazide **3d** was converted to the triazolidine **2b** (K_2CO_3, EtOH, reflux, 6 h, 81%) and then oxidized [$PhI(OAc)_2$, CH_2Cl_2, room temp., quantitative] to afford the triazoline **1b** as red crystals (m.p. 200–202 °C, decomp.). The reagent **1b**, being stable on handling at ambient temperature, can be stored in a freezer for a long time.

‡ The triazoline **1a** was not stable enough to be isolated.



The reaction of **1b** with the active vitamin D_3 metabolite **5** (dichloromethane, at room temperature) was fast (half-life of the order of seconds even in a solution as low as 10^{-7} M), efficient (nearly quantitative), and the product **6** was much more stable than the parent vitamin D. The detection limit of the adduct **6b** on HPLC with fluorescence detector (Ex 370 nm, Em 440 nm) *ca.* 0.1 fmol. All of the results showed that the reagent **1b** can be used to assay the active vitamin D metabolite.§

We are now developing a new convenient and precise method for assaying vitamin D metabolites in serum, using the new fluorigenic reagents **1a**, **b**.¶ Application to vitamin A metabolites is also under investigation.

We thank Biosensor Laboratories Co. Ltd. for financial support and Yuko Ikeda for her technical assistance.

Received, 18th April 1990; Com. 0/01727B

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§ The serum concentration of the metabolite **5** is *ca.* 10^{-10} M.

¶ Competitive binding assay using a receptor for the active metabolite **5**, which is the best method currently in use, lacks specificity, so it is troublesome and needs special techniques and its precision is poor. The detection limit of the method is *ca.* 10 fmol.