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Steroids

Coupling of biologically active steroids to conjugating arms through ether linkages for use in immunochemistry

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Abstract

Conjugation of haptens through ether linkages avoids leakage problems in immunoassays, but this procedure is not easily applied to most steroids that bear low reacting hydroxyls. A new technique allowing the ether coupling of biologically active steroids with conjugating arms in mild conditions compatible with thermosensitive protecting groups is presented. In the first step, the solvent (an aromatic hydrocarbon) was dehydrated by azeotropic distillation in a soxhlet apparatus using a cartridge filled with 0.3 nm and 0.4 nm molecular sieves. In this protected medium, a thallium steroid alkoxide was completely formed by reaction of the steroid with thallium ethoxide and by the continuous elimination of ethanol. The halogenated chain was then introduced into the same medium and reacted in the absence of moisture to give the ether. 17β-Hydroxy and 11α-hydroxy derivatives were involved in this reaction. The coupling was effective for all of the compounds tested after 2–36 h of reaction time and at temperatures between 80 and 140°C. The conjugates were at least 95% pure, and yields ranged from 15 to 95%.

Keywords: Ether linkages; Low reacting hydroxyls; Steroid conjugates; Thermosensitive compounds; Synthesis

1. Introduction

In a previous work, conjugates of steroids to penicillins through ester linkage were prepared for use in homogeneous immunoassays, but these bonds are very sensitive to chemical or enzymatic hydrolysis [1]. Ether linkages are suited to considerably reduce these leakage problems in immunoassays [2] but attempts at synthesizing ether bonds using the classic approach failed due to low reactivity and thermosensitivity of the reagents involved in this synthesis procedure. Nevertheless, the advantages resulting from stable steroid conjugates prompted us to investigate more efficient synthesis routes.

Ether formation is usually performed according to Williamson: an alcohol is reacted with a halogenated derivative [3]. Thallium I is claimed to accelerate the reaction rates and is often introduced as a thallium steroid alkoxide prepared in situ by reaction of the steroid and thallium ethoxide [4]. Unfortunately, this method has never been applied to steroids as 4-pregnen-11α-ol-3,20-dione (11α-hydroxyprogesterone), 4-androsten-17β-ol-3-one (testosterone), 4-estren-17β-ol-3-one (nandrolone) and 1,3,5(10)estratrien-3,17β-diol (estradiol) are involved in the control of the fertility and the metabolism. Other well-proven methods for ether synthesis involving phase-transfer or bis [acetylacetonato]nickel or silver trifluoromethanesulfonate catalysis have been no more successfully applied to these same steroids [5–7].

The aim of this work was to set up a synthesis route that combined mild reaction conditions to preserve thermosensitive reagents from degradation and a strong reactivity to allow for the conjugation of low reacting compounds.

2. Experimental

All reagents were of analytical or biochemical grade products purchased from Aldrich Chemical Company (Milwaukee, Wisconsin, USA), from Steraloids (Wilton, NH, USA), or from Merck (E. Merck, Darmstadt, Germany).

Columns filled with Silica gel 60 (230–400 mesh

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ASTM) from Merck (E. Merck, Darmstadt, Germany) or plates Alugram® Sil6/UV_{254} from Macherey-Nagel (Macherey-Nagel, Düren, Germany) were used for preparative and analytical chromatography.

Purity was estimated by thin layer chromatography. Equal volumes of the sample, twenty-fold and hundred-fold dilutions of the sample were deposited on the plate. After migration, the spots of the compound and the impurities were visually observed under a U-V lamp at 254 nm. The intensity of impurity spots in the sample were compared to the intensity of the spots of the main compound after twenty-fold and hundred-fold dilutions.

Mass spectra (MS) were obtained by electrospray ionization (ES) on a VG platform Fisons mass spectrometer [solvent:acetonitrile/water (50:50 v/v)] (Danvers, MA, USA). Infrared (IR) spectra of solutions (0.1% in tetrachlorethylene - pathlength: 500 μm) or pellets (0.4% dispersion in KBr) were recorded on a Perkin Elmer spectrum 2000 FTIR spectrophotometer (Beaconsfield, Bucks, England). Melting points were obtained on a Büchi B 530 apparatus (Flawil, Switzerland), and analytical data on a Carlo-Erba EA 1108 analyser (Milano, Italy).

Unless otherwise specified, solvent evaporations were carried out under reduced pressure (15 mmHg). Organic extracts were dried over anhydrous Na_2SO_4 and products over P_2O_5 under reduced pressure (1 mmHg).

2.1. Preparation of halogenated derivatives

*2.1.1. 4-(2-Bromoethoxy)benzaldoxime [**1**]*

Samples of 10 g (43.6 mmol) of 4-(2-bromoethoxy)benzaldehyde and of 6.06 g (87.2 mmol) of hydroxylamine hydrochloride were stirred for 2 h at 45°C in 200 ml of a mixture of methanol/water (50:50 v/v) adjusted to pH 4.5 with 4% aqueous NaOH. After removal of the methanol, the crude product was extracted with chloroform. After evaporation to dryness, the crystallized product was obtained from a mixture of chloroform/petroleum ether (b.p.: 40°C) (10:90 v/v) with a 92% yield. M.p.: 94°C. IR (cm^{-1}) (C_2Cl_4): 3595 (ν N-OH). (MS): (ES^+) m/z: 245.1 $(M+H)^+$. Anal. Calcd for $C_9H_{10}O_2NBr$: C, 44.29; H, 4.13; N, 5.73. Found: C, 44.58; H, 4.45; N, 5.75.

*2.1.2. 4-(2-Bromoethoxy)-3-chloro-benzohydroxamoyl chloride [**2**]*

A solution of 4 g (60 mmol) of chlorine in chloroform (150 ml) was poured into a refrigerated solution (−10°C) of 8 g (34.9 mmol) of [**1**] in 85 ml of an anhydrous mixture of 1,4-dioxane/chloroform (75:25 v/v). The mixture was left overnight at room temperature. After removal of the solvents, the residual oil was crystallized from a mixture of chloroform/petroleum ether (b.p.: 40°C) (5:95 v/v) in 78% yield. IR (cm^{-1}) (C_2Cl_4): 3570 (ν N-OH). (ES^-) m/z: 312.2 $(M-H)^-$. Anal. Calcd for $C_9H_8O_2NBrCl_2$: C, 34.54; H, 2.57; N, 4.48. Found: unstable.

*2.1.3. t-Butyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**3**]*

Refrigerated solutions (− 5°C) of 7 g (26.6 mmol) of [**2**] in acetonitrile (100 ml) and 5.89 g (30 mmol) of t-butyl acetoacetate potassium enolate in acetonitrile (60 ml) were slowly mixed and incubated overnight at the same temperature. The mixture was acidified with 0.01 N HCl to pH 6 and then extracted with chloroform. The product was purified by column chromatography using a petroleum ether (b.p.: 100–140°C)/ethyl-acetate mixture (75:25 v/v) as the mobile phase and crystallized from a mixture of chloroform/petroleum ether (b.p.: 40°C) (10:90 v/v) with a 76% yield. M.p.: 93°C. IR (cm^{-1}) (KBr): 1720 (ν C = O). (ES^+) m/z: 418.6 $(M+H)^+$. Anal. Calcd for $C_{17}H_{19}O_4NBrCl$: C, 49.01; H, 4.59; N, 3.36. Found: C, 49.28; H, 4.95; N, 3.58.

*2.1.4. 3-[4-(2-Bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylic acid [**4**]*

Samples of 4 g (10.46 mmol) of [**3**] and of 2.08 g (12 mmol) of p-toluenesulfonic acid were stirred in 70 ml of a mixture of toluene/nitromethane (65: 35 v/v) at 25°C for 6 h. The suspension was then diluted with chloroform and washed with water. After evaporation, the residue was crystallized from a mixture of toluene/petroleum ether (b.p.: 100–140°C) (10: 90 v/v) with a 81% yield. M.p.: 163°C. IR (cm^{-1}) (KBr): 1689 (ν C = O). (ES^-) m/z: 360.3 $(M-H)^-$. Anal. Calcd for $C_{13}H_{11}O_4NBrCl$: C, 43.30; H, 3.07; N, 3.88. Found: C, 43.54; H, 3.41; N, 3.99.

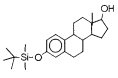
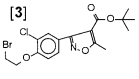
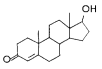
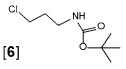
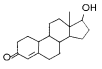
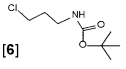
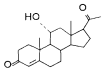
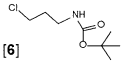
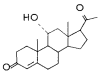
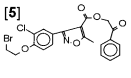
*2.1.5. Phenacyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**5**]*

Samples of 364 mg of potassium fluoride (6.27 mmol), of 1.24 g of α-bromoacetophenone (6.27 mmol), and of 1.9 g (5.2 mmol) of [**4**] were stirred at 25°C in 55 ml of N,N-dimethylformamide for 3 h. After concentration under reduced pressure (1 mmHg), the residue was diluted with chloroform and washed with water. The product was chromatographed as described for [**3**] and crystallized from a mixture of toluene/petroleum ether (b.p.: 40°C) (10:90 v/v) with a 76% yield. M.p.: 119°C. IR (cm^{-1}) (KBr): 1725 (ν C = O); 1692 (ν C = O). (ES^+) m/z: 481.3 $(M+H)^+$. Anal. Calcd for $C_{21}H_{17}O_5NBrCl$: C, 52.69; H, 3.58; N, 2.92. Found: C, 52.82; H, 3.72; N, 2.99.

*2.1.6. t-Butyl N-(3-chloropropyl) carbamate [**6**]*

Samples of 2.31 g (22.91 mmol) of chloropropylamine hydrochloride and of 5 g (22.91 mmol) of ditert-butyl dicarbonate, dissolved in a mixture of tetrahydrofuran/water (50:50 v/v), adjusted at pH 8.5 with 4% aqueous NaOH were stirred for 4 h at room temperature. The solution was acidified to pH 2 using 0.1 N HCl and then extracted with chloroform. After evaporation of the solvent, the product was crystallized from a mixture of chloroform/petroleum

Table 1
Reacting compounds, reaction conditions, and main analytical characteristics of final products

Steroid	Halogenated derivative	Chromatography		M.S.	IR cm^{-1}	Reaction time (Solvent)	Analytical calculations (%)		Melting point (°C)	Yield %
		Thin layer (Rf)	Col.				Theor.	Found		
	 [3]	toluene. 1 ethyl acetate 1 HOAc 0.01 (0.81)	+	721.4 $(M-H)^-$ 649.4	ν CO: 1725 ν O–Si: 1257	24 H (Benzene)	C: 68.24 H: 7.77 N: 1.94	C: 68.31 H: 7.86 N: 2.00	143	22
	 [6]	toluene. 1 ethyl acetate 2 HOAc 0.01 (0.78)	+	445.6 $(M-H)^+$ 355.4	ν NH: 3459 ν CO: 1729 ν CO: 1681	12 H (Benzene)	C: 72.61 H: 9.93 N: 3.13	C: 72.92 H: 10.21 N: 3.25	155	72
	 [6]	toluene. 1 ethyl acetate 2 HOAc 0.01 (0.77)	+	431.6 $(M-H)^+$	ν NH: 3460 ν CO: 1729 ν CO: 1679	36 H (Toluene)	C: 72.36 H: 9.57 N: 3.24	C: 72.51 H: 9.89 N: 3.19	127	15
	 [6]	toluene. 1 ethyl acetate 2 HOAc 0.01 (0.61)		488.3 $(M-H)^+$	ν NH: 3454 ν CO: 1726 ν CO: 1711 ν CO: 1683	12 H (Benzene)	C: 71.43 H: 9.22 N: 2.87	C: 71.58 H: 9.35 N: 3.24	124	74
	 [5]	toluene. 2 ethyl acetate 1 HOAc 0.01 (0.88)	+	343.4 401.3	ν CO: 1726 ν CO: 1708 ν CO: 1691 ν CO: 1673	14 H (Xylene)	C: 69.27 H: 6.3 N: 1.92	C: 69.65 H: 6.71 N: 2.13	148	55

ether (b.p.: 40°C) (20:80 v/v) with a 92% yield. M.p.: 32°C. IR (cm^{-1}) (C_2Cl_4): 1723 (ν C = O); 3464 (ν N-H); (ES^+) m/z: 193.8 $(M+H)^+$. Anal. Calcd for $C_8H_{16}O_2$ NCl: C, 49.61; H, 8.32; N, 7.23. Found: C, 49.84; H, 8.56; N, 7.46.

2.2. General procedure for coupling steroids to unstable alkylhalides (Table 1)

A solution of steroid (1 mmol) in 100 ml of an aromatic hydrocarbon (see Table 1) was heated under reflux in a soxhlet apparatus well protected from light by aluminium foil applied to the balloon flask. The cartridge of the apparatus was filled with a mixture of 3 Å and 4 Å molecular sieves in equal proportions. After 60 min, 1.2 mmol of thallium ethoxide was added to the boiling solution, and the reaction was continued for an additional 2 h period. After cooling to room temperature, a solution of 1.0 mmol of halogenated derivative in 5 ml of the same solvent was added, and the mixture was boiled again. When no further evolution of the reaction seemed to occur, as determined by thin layer chromatography, the solvent was evaporated, and the residue was dissolved in 50 ml of chloroform. The organic phase was washed with water, dried, and evaporated. The oily residue was crystallized from a mixture of toluene/petroleum ether (b.p.: 40°C) (5:90 v/v) after an optional purification (marked '+' in Table 1) by column chromatography using the mobile phase defined in Table 1.

3. Results

All of the synthesized conjugating arms and all of the ether-linked steroid conjugates prepared by this technique demonstrated the expected characteristics in either elemental analysis, mass spectrometry, or Fourier transform infrared spectrometry (Table 1).

High yields, ranging from 76 to 92%, were obtained for each step of the synthesis procedure of conjugating arms (overall yields: 33–92%), while steroid conjugates were prepared in yields ranging from 15 to 75%, depending on temperature, reaction time, and steroid reactivity (Table 1). Each conjugate was purified until any impurity spot did not show intensity greater than that of the hundred-fold dilution of the tested compound. In that way, the overall purity of the conjugate could be estimated to be better than 95%.

Bromoethoxyphenylisoxazolecarboxylic derivatives were prepared following the procedure developed and discussed previously for carboxyphenylisoxazolecarboxylic derivatives [1]. However, during the preparation of 4-(2-bromoethoxy)-benzohydroxamoyl chloride, the phenyl ring was substituted at the 3-position by a chlorine atom. This substitution, detected by mass spectrometry, was confirmed and positioned by an X-ray diffraction study of t-butyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**3**] [8]. The derivative resulting from that reaction was the unstable 4-(2-bromoethoxy)-3-chlorobenzohydroxamoyl chloride [**2**].

Only protected estradiol reacted with t-butyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-

carboxylate [**3**] while 11α-hydroxyprogesterone that did not react with [**3**] gave a conjugate to phenacyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**5**]. 11α-Hydroxyprogesterone also reacted with t-butyl N-(3-chloropropyl) carbamate [**6**] in higher yield and this last reagent was also conjugated to testosterone and nandrolone. In all of the reactions considered, the yield reached a maximum after a reaction time which varied from one compound to another. As monitored by thin layer chromatography, the different reagents were progressively consumed and the final product itself was altered when the reaction was continued beyond the time recommended.

4. Discussion

In a previous work, steroids were coupled to carbenicillin and oxacillin side chains through ester linkages [1]. The aim was to prepare strong β-lactamase inhibitors whose effect could be suppressed after antibody–steroid conjugate interaction. From this point of view, the best compounds were those having the shortest possible conjugating arm (Steroid-O-CO-Oxacillin). In order to preserve the conjugates from serum esterase activity, ether linkages are a possible alternative. t-Butyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**3**] was first selected for its close similarity to the ester linked compounds previously described [1] and the easy removal of the t-butyl group. Unfortunately, [**3**] failed to react with most of the investigated compounds at temperatures compatible with the t-butyl group (solvents: benzene or toluene). Phenacyl 3-[4-(2-bromoethoxy)-3-chlorophenyl]-5-methylisoxazole-4-carboxylate [**5**] was prepared with the aim of increasing stability against decarboxylation, thus allowing conjugation at higher temperatures (solvent: xylene). t-Butyl N-(3-chloropropyl) carbamate [**6**] was selected for the high reactivity of its halogenate although the length of the coupling arm should be significantly increased after subsequent reaction of the amine to the carboxyoxacillin.

For the steroid coupling, the optimal reaction conditions were set out in the silylation of estradiol on its phenol. A compound identical to the product described by Top et al. [9] was obtained with a very good yield (95%). This operating mode was then applied to the O - alkylation of four steroids: silylated estradiol, nandrolone, testosterone, and 11α-hydroxyprogesterone. Silylated estradiol reacted with [**3**] in benzene, and the yield reached a maximum of 22% after 24 h reaction time, but no reaction occurred between [**3**] and the other steroids in this solvent. In xylene, trace amounts of progesterone conjugate were formed in the early phase of the reaction, but the conjugate was destroyed as soon as it was formed. Using the less thermosensitive compound [**5**], the corresponding conjugate was obtained with a 55% yield after 14 h, but neither testosterone nor nandrolone did react with [**5**]. A more reactive compound [**6**], although potentially much less interesting in immunochemistry, was tested on 11α-hydroxyprogesterone (benzene, 12 h, 74% yield). The yield increase obtained at lower temperature seemed to be due to the greater reactivity of [**6**] more probably than to the better formation of the thallium steroid alkoxide, and prompted us to apply this reaction for coupling to testosterone (benzene, 12 h, 72% yield), and nandrolone (toluene, 36 h, 15% yield).

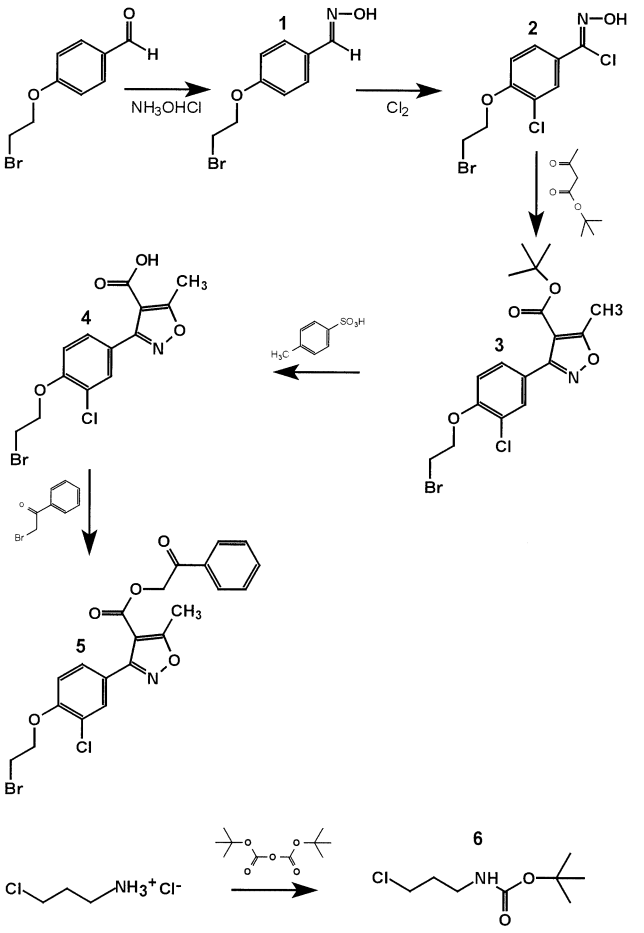


Fig. 1. Synthesis routes for the preparation of conjugating arms for coupling to steroids. Preparation of halogenated derivatives.

The halogenated derivatives included in the study, except for [**5**], were unstable by decarboxylation, and the steroids selected, as secondary alcohols bearing long alkyl chains, were poor nucleophiles. Despite these unfavorable properties of the reagents involved in the conjugation, a coupling was obtained with all of the steroids tested. The continuous elimination of moisture traces by distillation over the molecular sieves certainly contributed to maintain the best conditions during the rather long reaction time. The maximum yield varied inversely as a function of the reaction time and resulted from a competition between the kinetics of the conjugation and the degradation rate of the final products.

In conclusion, the proposed method is an efficient approach to the conjugation of steroid by ether formation with halogenated derivatives protected by thermosensitive groups (Fig. 1).

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