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Synthesis of 4-Carboxymethylthio-Substituted Steroids for Enzyme Immunoassay

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The 4-carboxymethylthio derivatives 8 and 13, new haptens of betamethasone (2) and dexamethasone (1), were prepared for application to enzyme immunoassay.

Keywords hapten; enzyme immunoassay; carboxymethylthiolation; betamethasone; dexamethasone; 4-(carboxymethylthio)betamethasone; 4-(carboxymethylthio)dexamethasone

It is well known that prolonged administration of some synthetic adrenocortical agents such as dexamethasone (**1**) and betamethasone (**2**) can have serious adverse effects. As one of the methods to determine the amounts of these agents in serum, enzyme immunoassay has been most widely and generally used in hospitals and clinical research laboratories. This assay requires the preparation of effective antibody and enzyme-labeled antigen, thus necessitating the synthesis of an appropriate steroid derivative carrying a substituent capable of binding with the protein concerned.

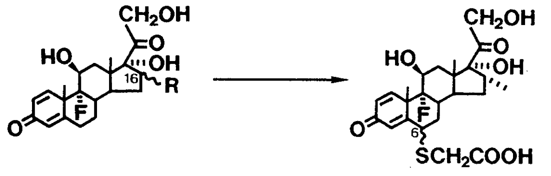
Though there is an immunoassay for **2** using the 3-(*O*-carboxymethyl)oxime[1] as the hapten, an improved method using a more efficient antibody with higher specificity is required. For this purpose, we assumed that a suitable substituent should be introduced at C-4[2,3] or C-6[3,4] of **2**. In the case of **1**, Shibata *et al.*[5] had reported that the reaction of the 6-bromide with thioglycolic acid afforded the corresponding 6-substituted product **3**. Therefore we carried out the reaction of the betamethasone derivative with thioglycolic acid according to the procedure described by Shibata *et al.*[5]

Bromination of the bismethylenedioxybetamethasone (**4**)[6] in dioxane afforded a mixture of two bromides **5** and **6** in 69% and 7% yields, respectively. These compounds were separated and their structures were established on the basis of spectral evidence. Their mass spectra (MS) exhibited the molecular and isotopic ion peaks at *m*/*z* 512 and 514 with an equivalent ratio, thus showing the introduction of a bromine atom into the molecule. In the proton nuclear magnetic resonance (^{1}H-NMR) spectra, the signal of hydrogen at C-6 appeared as a broad triplet ($J=3.5$ Hz) in **5** while as a double doublet of doublets ($J=12$, 6, and 2 Hz) in **6** as the result of allylic coupling with the olefinic hydrogen at C-4. Furthermore, the signals of hydrogen at C-8 and the methyl group at C-10 in **5** appeared at lower field than those of the starting compound **4** due to the influence of the bromine atom at C-6. On the other hand, the signal of the hydrogen at C-4 in **6** appeared at low field, thus establishing that **5** is the 6β-bromide while **6** is the 6α-bromide.

Treatment of the bromides **5** and **6** with thioglycolic acid in a potassium hydroxide–methanol solution at reflux temperature afforded the identical product **7** in 55% and 20% yields, respectively. Compound **7** showed a molecular ion peak at *m*/*z* 524 and carboxyl group absorption in its infrared (IR) spectrum and ^{1}H-NMR spectrum, in which a peak due to olefinic hydrogen at C-4 disappears while a peak due to olefinic hydrogen at C-2 appears as a doublet ($J=10$ Hz) as the result of disappearance of a W-shaped long-range coupling between two hydrogens at C-2 and C-4 in addition to the appearance of a peak due to a newly introduced methylene group in the side chain. Finally, hydrolysis of **7** in 50% acetic acid brought about deprotection to give 4-(carboxymethylthio)betamethasone (**8**) in 54% yield. Its ^{1}H-NMR data clearly showed that a substituent was present at C-4, as summarized in Table II. Therefore, it became necessary to clarify the reason for the different result in the case of **1**.

Thus, we have reinvestigated the reaction of **1** according to the reported procedure.[5] Bromination of the bismethylenedioxydexamethasone (**9**) was carried out as in the case of **2** and two bromides **10** and **11** were obtained in 60% and 2% yields, respectively. Their structures were established from spectral evidence as in the case of **5** and **6**.

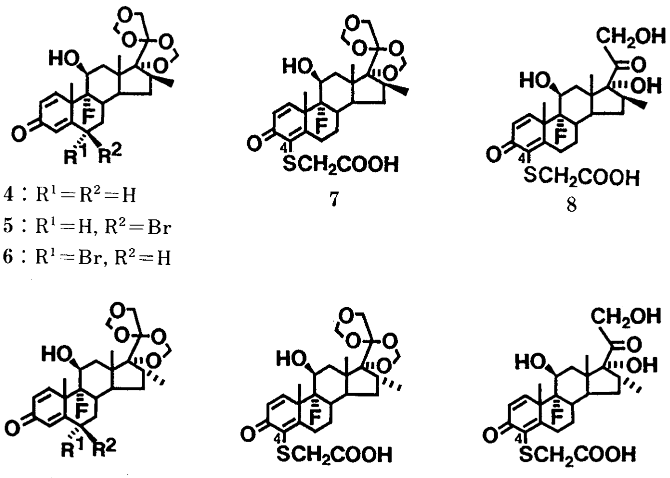
When the 6β-bromide **10** was treated with thioglycolic acid according to Shibata's procedure,[5] the carboxymethylthio derivative **12**, which had a substituent at C-4



1: R=16α-Me dexamethasone
2: R=16β-Me betamethasone

3: 6-(carboxymethylthio)-dexamethasone

Chart 1



4: $R^1=R^2=H$
5: $R^1=H$, $R^2=Br$
6: $R^1=Br$, $R^2=H$

7

8

9: $R^1=R^2=H$
10: $R^1=H$, $R^2=Br$
11: $R^1=Br$, $R^2=H$

12

13

Chart 2

Table I. Reaction of the Bromides **10** and **11** with Thioglycolic Acid

Substrate	Molar ratio of substrate	:	thioglycolic acid	:	base	Solvent	Temperature$^{a)}$	Time	Yields (%) of products **12**	**9**
10	1	:	1.6	:	4 (KOH)	MeOH	r.t.	4 h	31	46
10	1	:	5	:	12 (KOH)	MeOH	r.t.	80 min	42	51
10	1	:	1.6	:	4 (NaOMe)	Dioxane–MeOH	r.t.	75 min	55	20
10	1	:	5	:	12 (NaOMe)	Dioxane–MeOH	r.t.	35 min	72	16
11	1	:	5	:	12 (NaOMe)	Dioxane–MeOH	Reflux	5 h	46	16

a) r.t. = room temperature.

Table II. ^{1}H-NMR Data (δ; J in Hz) for Carboxymethylthio Derivatives **8**, **13** (in $CDCl_3$–CD_3OD, 200 MHz), and **3** (in DMSO-d_6, 90 MHz)[5)]

	8	**13**	**3**
1-H	7.38 (d, $J=10$)	7.38 (d, $J=10$)	7.26 (d, $J=10$)
2-H	6.42 (d, $J=10$)	6.45 (d, $J=10$)	6.1 (dd, $J=10$, 2)
4-H	—	—	5.95 (s)
6α-H	3.75 (br d, $J=14$)	3.78 (br d, $J=14$)	
6β-H	2.56 (td, $J=14$, 6)	2.51 (td, $J=14$, 6)	
11-H		4.27 (m)	4.1 (br s)
21-H_2	4.34 and 4.50 (ABq, $J=20$)	4.29 and 4.61 (ABq, $J=20$)	4.31 and 4.60 (ABq, $J=20$)
10-Me	1.61 (s)	1.61 (s)	1.55 (s)
13-Me	1.08 (s)	1.02 (s)	0.98(s)
16-Me	1.13 (d, $J=7$)	0.90 (d, $J=7$)	0.89 (d, $J=7$)
SCH_2CO	3.30 (s)	3.42 and 3.49 (ABq, $J=15$)	

instead of C-6 as they had claimed, was obtained along with the hydrogenated product **9**. The structure of the 4-carboxymethylthio derivative **12** was established as in the case of **7** from spectral evidence. Even upon thorough scrutiny of the reaction mixture, none of the 6-carboxymethylthio derivative was detected. In order to exclude the possibility of the effect of a subtle change in the reaction conditions, the reaction was thoroughly reinvestigated under various conditions as shown in Table I. However, only the product **12** was obtained from the bromides **10** and **11**.

Deprotection of **12** with 60% formic acid[7)] afforded the 4-carboxymethylthio derivative **13**, for which the ^{1}H-NMR data are summarized in Table II. The comparison of the spectral data of 4-(carboxymethylthio)dexamethasone (**13**) with those of the 6-substituted derivative **3** described by Shibata *et al.*[5)] reveals a clear difference.

Thus, we conclude that we have obtained only the 4-substituted derivatives **8** and **13** from **2** and **1** by carboxymethylthiolation, and we could not reproduce the result reported by Shibata *et al.*[5)]

The application of these newly synthesized 4-carboxymethylthio derivatives **8** and **13** of **2** and **1** to the enzyme immunoassay has been successful and the results will be published in a separate paper.

Experimental

All melting points were measured on a micro hot-stage apparatus (Yanagimoto) and are uncorrected. ^{1}H-NMR spectra were measured with a Varian XL-200 (200 MHz) instrument using tetramethylsilane as an internal reference, IR spectra were taken in nujol mulls with Hitachi 215 and 270-30 machines. MS were taken with a Hitachi M-80 spectrometer. Ultraviolet (UV) spectra were obtained on a Shimadzu UV-190 spectrophotometer in methanol and optical rotations on a JASCO DIP-181 digital polarimeter at room temperature. Preparative thin layer chromatography (TLC) was carried out on precoated plates of silica gel (Kieselgel 60 F_{254}, Merck). Extracts from the reaction mixture were dried over anhydrous sodium sulfate and evaporated under reduced pressure.

Bromination of Bismethylenedioxybetamethasone (4) A solution of bromine (0.024 ml) in dioxane (3.6 ml) was slowly added dropwise to a solution of **4**[6)] (63 mg) in dioxane (2 ml) at room temperature and the mixture was stirred for 1 h. The solvent was evaporated off to obtain the residue, which was treated with saturated $NaHCO_3$ under ice cooling and extracted with diethyl ether. The combined extracts were washed with brine, 0.5 M $Na_2S_2O_7$ and finally with brine, and dried. Evaporation of the solvent afforded the residue, which was crystallized from diethyl ether–acetone to afford (6β,11β,16β)-6-bromo-9-fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis(oxy)]pregna-1,4-dien-3-one (**5**) (37 mg). The mother liquor afforded upon preparative TLC (benzene–ethyl acetate, 5 : 3) the products **5** (14 mg) and (6α,11β,16β)-6-bromo-9-fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis(oxy)]pregna-1,4-dien-3-one (**6**) (5 mg). The yield of **5** reached 69% while that of **6** was 7%.

5: mp 195—197 °C (dec.) (from Et_2O), colorless plates. $[\alpha]_D$ −40.0° ($c=0.50$, MeOH). UV λ_{max} nm: 240. EI-MS m/z: 512 (M^+), 514 ($M^+ + 2$). IR ν_{max} cm^{-1}: 3590, 3320, 1665, 1630, 1615. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.14 (3H, d, $J=7$ Hz, 16-Me), 1.22 (3H, s, 13-Me), 1.92 (3H, s, 10-Me), 2.80 (1H, m, 8-H), 4.06 and 4.28 (2H, ABq, $J=9$ Hz, 21-H_2), 4.37 (1H, m, 11-H), 5.13 (1H, br t, $J=3.5$ Hz, 6α-H), 4.93—5.18 (4H, m, $OCH_2O \times 2$), 6.36 (1H, br s, 4-H), 6.37 (1H, br d, $J=10$ Hz, 2-H), 7.39 (1H, d, $J=10$ Hz, 1-H). *Anal.* Calcd for $C_{24}H_{30}BrFO_6$: C, 56.14; H, 5.89. Found: C, 56.24; H, 5.99.

6: mp 234—236 °C (dec.) (from Et_2O), colorless plates. $[\alpha]_D$ −58.8° ($c=0.51$, MeOH). UV λ_{max} nm: 240. EI-MS m/z: 512 (M^+), 514 ($M^+ + 2$). IR ν_{max} cm^{-1}: 3530, 1665, 1630. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.12 (3H, d, $J=7$ Hz, 16-Me), 1.15 (3H, s, 13-Me), 1.61 (3H, s, 10-Me), 4.05 and 4.26 (2H, ABq, $J=9$ Hz, 21-H_2), 4.27 (1H, m, 11-H), 5.14 (1H, ddd, $J=12$, 6, 2 Hz, 6β-H), 4.92—5.16 (4H, m, $OCH_2O \times 2$), 6.39 (1H, dd, $J=10$, 2 Hz, 2-H), 6.75 (1H, t, $J=2$ Hz, 4-H), 7.30 (1H, d, $J=10$ Hz, 1-H). *Anal.* Calcd for $C_{24}H_{30}BrFO_6$: C, 56.14; H, 5.89. Found: C, 56.04; H, 5.84.

[[(11β,16β)-9-Fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis(oxy)]-3-oxopregna-1,4-dien-4-yl]thio]acetic Acid (7) i) From **5**: Thioglycolic acid (0.7 ml) was added to a solution of **5** (236 mg) dissolved in methanol (94 ml). The resulting solution was heated under reflux while adding a solution of 6% KOH–methanol (19 ml) dropwise. The reaction mixture was refluxed for 72 h, then the solvent was evaporated off and the residue was taken up in water and extracted with chloroform. The aqueous layer was acidified with 10% HCl to pH 1 and then extracted with chloroform. The extract was washed with water, dried and evaporated. The residue was crystallized with diethyl ether–petroleum ether to afford the product **7** (133 mg, 55%) as colorless crystals, mp 230—231.5 °C (dec.) (from Et_2O). $[\alpha]_D$ +20.0° ($c=0.50$, MeOH). UV λ_{max} nm: 218, 240. EI-MS m/z: 524 (M^+). IR ν_{max} cm^{-1}: 3700—2500, 1655, 1635—1550. ^{1}H-NMR ($CDCl_3$) δ: 1.12 (3H, d, $J=7$ Hz, 16-Me), 1.14 (3H, s, 13-Me), 1.60 (3H, s, 10-Me), 3.37 and 3.46 (2H, ABq, $J=15$ Hz, SCH_2CO), 3.74 (1H, br d, $J=14$ Hz, 6α-H), 4.03 and 4.24 (2H, ABq, $J=10$ Hz, 21-H_2), 4.30 (1H, m, 11-H), 4.90—5.17 (4H, m, $OCH_2O \times 2$), 6.52 (1H, d, $J=10$ Hz, 2-H), 7.39 (1H, d, $J=10$ Hz, 1-H). *Anal.* Calcd for $C_{26}H_{33}FO_8S$: C, 59.52; H, 6.34. Found: C, 59.34; H, 6.44.

ii) From **6**: Thioglycolic acid (0.06 ml) was added to a solution of **6** (20 mg) dissolved in methanol (8 ml) and 6% KOH–methanol solution (1.6 ml) was added dropwise to the resulting solution at refluxing temperature. The reaction mixture was refluxed for a further 4 h and worked up in the same manner as above. The crude product was purified by preparative TLC (chloroform–methanol, 6 : 1) to give **7** (4 mg, 20%).

[[(11β,16β)-9-Fluoro-11,17,21-trihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-4-yl]thio]acetic Acid [4-(Carboxymethylthio)betamethasone] (8)

A solution of **7** (80 mg) in 50% acetic acid (18 ml) was heated at 100 °C under a nitrogen stream for 5 h. The solvent was evaporated off to leave the residue, which was purified by preparative TLC (chloroform–methanol, 5 : 3) to afford the product **8** (40 mg, 54%) as a white powder, mp 230 °C (dec.) (ethyl acetate–isooctane). UV $\lambda_{\max}$ nm: 215, 240. FD-MS *m/z*: 483 ($M^+ + 1$). IR $\nu_{\max}$ cm^{-1}: 3700—2500, 1710, 1655, 1580. ^1H-NMR: Table II. *Anal.* Calcd for $C_{24}H_{31}FO_7S \cdot 3/2H_2O$: C, 56.56: H, 6.73. Found: C, 56.79; H, 6.72.

Bromination of Bismethylenedioxydexamethasone (9) In the same way as described for the bromination of **4**, bromination of **9**[7] (440 mg) in dioxane (48 ml) with bromine (0.078 ml) in dioxane (12 ml) followed by chromatography on silica gel with chloroform as eluent gave (6β,11β,16α)-6-bromo-9-fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis-(oxy)]pregna-1,4-dien-3-one (**10**) (247 mg). Additional crops of the products **10** (67 mg) and (6α,11β,16α)-6-bromo-9-fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis(oxy)]pregna-1,4-dien-3-one (**11**) (11 mg) were obtained by preparative TLC (benzene–ethyl acetate, 5 : 3). Total yields of **10** and **11** were 60% and 2%, respectively.

10: mp 208—211 °C (dec.) (from MeOH), colorless cubes. $[\alpha]_D$ −15.9° (*c* = 0.44, MeOH). UV $\lambda_{\max}$ nm: 245. CI-MS *m/z*: 512 (M^+), 514 ($M^+ + 2$). IR $\nu_{\max}$ cm^{-1}: 3335, 1660, 1620. ^1H-NMR ($CDCl_3$) δ: 0.96 (3H, d, *J* = 7 Hz, 16-Me), 1.24 (3H, s, 13-Me), 1.90 (3H, s, 10-Me), 2.74 (1H, m, 8-H), 4.03 (2H, s, 21-H_2), 4.42 (1H, m, 11-H), 4.98—5.27 (5H, m, $OCH_2O \times 2$ and 6α-H), 6.36 (2H, m, 2-H and 4-H), 7.30 (1H, d, *J* = 10 Hz, 1-H). *Anal* Calcd for $C_{24}H_{30}BrFO_6$: C, 56.14; H, 5.89. Found: C, 56.18; H, 5.84.

11: mp 241—244 °C (dec.) (from MeOH), colorless cubes. $[\alpha]_D$ −40.1° (*c* = 0.47, MeOH). UV $\lambda_{\max}$ nm: 239. CI-MS *m/z*: 512 (M^+), 514 ($M^+ + 2$). IR $\nu_{\max}$ cm^{-1}: 3400, 1665, 1620. ^1H-NMR ($CDCl_3$) δ: 0.94 (3H, d, *J* = 7 Hz, 16-Me), 1.17 (3H, s, 13-Me), 1.58 (3H, s, 10-Me), 4.01 (2H, s, 21-H_2), 4.33 (1H, m, 11-H), 4.95—5.24 (5H, m, $OCH_2O \times 2$ and 6β-H), 6.38 (1H, dd, *J* = 10, 2 Hz, 2-H), 6.75 (1H, t, *J* = 2 Hz, 4-H), 7.17 (1H, d, *J* = 10 Hz, 1-H). *Anal.* Calcd for $C_{24}H_{30}BrFO_6$: C, 56.14; H, 5.89. Found: C, 56.23; H, 5.88.

Carboxymethylthiolation of the Bromides 10 and 11 Thioglycolic acid (1.6 or 5 mol eq) and a base 4 or 12 mol eq) were added to a solution of **10** or **11** (30 mg) in methanol (4 ml) or dioxane–methanol (1 : 1, 2 ml) and the solution was treated at the temperature and for the time described in the relevant entry in Table I. After the reaction, the solvent was evaporated off. The residue was treated with water and extracted with chloroform. The combined chloroform extracts were washed with water, dried and evaporated. The resulting residue was subjected to preparative TLC (chloroform–methanol, 100 : 1) to give **9**. The aqueous layer was acidified with 10% HCl to pH 1 and then extracted with chloroform. The combined extracts were washed with water, dried, and evaporated to give the residue, which was purified by preparative TLC (chloroform–methanol, 4 : 1), affording [[(11β,16α)-9-fluoro-11-hydroxy-16-methyl-17,20,20,21-bis[methylenebis(oxy)]-3-oxopregna-1,4-dien-4-yl]thio]acetic acid (**12**) as a white powder, mp 188—191 °C (from Et_2O). $[\alpha]_D$ +35.8° (*c* = 0.50, MeOH). UV $\lambda_{\max}$ nm: 216, 240. EI-MS *m/z*: 524 (M^+). IR $\nu_{\max}$ cm^{-1}: 3700—2500, 1715, 1655, 1625, 1575. ^1H-NMR ($CDCl_3$) δ: 0.95 (3H, d, *J* = 7 Hz, 16-Me), 1.18 (3H, s, 13-Me), 1.59 (3H, s, 10-Me), 3.40 and 3.48 (2H, ABq, *J* = 15 Hz, SCH_2CO), 3.73 (1H, br d, *J* = 14 Hz, 6α-H), 4.01 (2H, s, 21-H_2), 4.29 (1H, m, 11-H), 4.97—5.27 (4H, m, $OCH_2O \times 2$), 6.51 (1H, d, *J* = 10 Hz, 2-H), 7.39 (1H, d, *J* = 10 Hz, 1-H). *Anal.* Calcd for $C_{26}H_{33}FO_8S \cdot H_2O$: C, 57.55; H, 6.50. Found: C, 57.38; H, 6.33.

[[(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-4-yl]thio]acetic Acid [4-(Carboxymethylthio)dexamethasone] (13) Compound **12** (32 mg) was dissolved in 60% formic acid (1 ml) and the resulting solution was heated under a nitrogen stream at 100 °C for 30 min. After the reaction, the reaction mixture was diluted with water and extracted with ethyl acetate. The combined extracts were washed with water, dried and evaporated to give the residue, which was subjected to preparative TLC (chloroform–methanol, 3 : 1) to afford **13** (19 mg, 65%) as a white powder, mp 195 °C (dec.) (from Et_2O). $[\alpha]_D$ +117.4° (*c* = 0.45, MeOH). UV $\lambda_{\max}$ nm: 212, 239. FD-MS *m/z*: 483 ($M^+ + 1$). IR $\nu_{\max}$ cm^{-1}: 3700—2500, 1710, 1655, 1620, 1570. ^1H-NMR: Table II. *Anal.* Calcd for $C_{24}H_{31}FO_7S \cdot 2H_2O$: C, 55.58; H, 6.80. Found: C, 55.66; H, 6.68.

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