The Hexacarbonyl(ethyne)dicobalt Unit: An Androgen Tag

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The interaction of estrogens and androgens with their corresponding receptors is known to play an important role in cancers of the breast and prostate. This paper reports the synthesis, characterization, and biochemical properties of a novel organometallic complex derived from 17α-ethynyltestosterone, namely hexacarbonyl{μ-[(20,21-η:20,21-η)-(17α)-17-hydroxypregn-4-en-20-yn-3-one}dicobalt ([$Co_2(CO)_6$(17α-ethynyltestosterone)]). The crystal and molecular structure of this compound was determined by single-crystal X-ray diffraction: it crystallizes in the monoclinic space group with $a = 24.6600(18)$ Å, $b = 12.9188(10)$ Å, $c = 26.3573(19)$ Å, $\beta = 108.651(2)^\circ$, and $Z = 12$. A biochemical study showed that the compound is still recognized by the androgen receptor even when the relative binding affinity (*RBA*) is quite low (0.5%). This finding can be explained by the recently published 3D structure of the androgen receptor that shows that its binding site cannot accommodate a bulky substituent at the 17α position of the steroid.

Introduction. – The role of estrogen receptors (ER) and their interactions with various estrogenic hormones (mainly estradiol = estra-1,3,5(10)-triene-3,17-diol), giving rise to an activated dimeric ER/estrogen complex that is capable of interacting with DNA and inducing mammary cells to proliferate, is now fairly well-understood [1]. The amino acid sequence of the entire ER has been inferred, and 2D-NMR and X-ray crystallographic studies have elucidated the details of the hormone-binding domain as well as the DNA-binding domain.

Furthermore, for ER-positive breast-cancer tumors, hormone therapy is the major therapeutic choice, and tamoxifen, the antiestrogen the most widely used in adjuvant therapy, represents a milestone in this protocol [2]. The situation is far more complex for prostate cancer. Testosterone (=(17β)-17-hydroxyandrost-4-en-3-one), which is produced by the testes, is the circulating androgen with the highest concentration in blood. However, the predominant androgen found in prostate cells is 5α-dihydrotestosterone (5α-DHT), obtained by reduction of testosterone by the NADPH-dependent enzyme 5α-reductase. Both testosterone and DHT are recognized by the androgen receptor (AR), but, in target cells, the active steroid is DHT [3]. Human AR possesses an estimated molecular mass of *ca.* 98000 Da and contains about 900 amino acids. Its mechanism of action is almost identical to that of the other members of the nuclear receptor superfamily, and the overall sequence homology with other steroid receptors ranges up to 54% [4]. As for breast cancers, some antihormones, in this case antiandrogens (cyproterone acetate, megestrol acetate, flutamide), have shown their

usefulness in the treatment of hormone-dependent prostate cancers [5]. However, their use is less widespread. Moreover, while a number of estradiol-organometallic derivatives have so far been prepared and tested for ER recognition [6–9], there is only one very recent report in the literature dealing with an androgen labelled with a metallic tag, namely the '19-nortestosterone-17α-ethynylferrocene' (=(17α)-21-ferrocenyl-17-hydroxy-19-norpregn-4-en-20-yn-3-one) [10]; 19-nortestosterone derived from testosterone by removal of the Me(19) group shows an increased affinity for the AR as well as for the progestinic receptor (PgR) with respect to testosterone. The only biological test available for AR interaction with '19-nortestosterone-17α-ethynylferrocene' was a co-transfection assay in prostate cancer PC-3 cells [11].

We have employed as androgen the prototypical 17α-ethynyltestosterone (=(17α)-17-hydroxypregn-4-en-20-yn-3-one; ET), since a number of organometallic derivatives of 17α-ethynylestradiol (=(17α)-19-norpregna-1,3,5(10)-trien-20-yne-3,17-diol) have been published and are known to retain a reasonable or even high affinity for the ER [6–8]. Interestingly enough, the 17α-ethynyl group reduces the binding to serum proteins that can lower the ready availability of hormones [12]. As organometallic tag, we have chosen the hexacarbonyldicobalt moiety, since its coordination to an alkyne chain is straightforward. Furthermore, (alkyne)carbonylcobalt complexes of salicylic acid have been reported to exhibit cytotoxicity against lung-carcinoma cell lines (IC_{50} = 4–6 μM) [13]. Thus, if the resulting hexacarbonyl(17α-ethynyltestosterone)dicobalt ([$Co_2(CO)_6$(ET)]; *Fig. 1*) is still recognized by its specific receptor, it could be used to convey a potential cytotoxic moiety into androgen-dependent prostate tumors. In addition, it has been demonstrated that carbonylmetal complexes exhibit extremely intense IR bands in the 2200–1800 cm^{-1} region and can be used as tracers in a non-isotopic immunoassay, namely CMIA (carbonylmetallo immuno assay) [14]. Furthermore, the dimetallic tag provides an independent, well-behaved reduction process that can be quantified by classical electrochemical techniques such as LSV (linear-sweep voltammetry) or SWV (square-wave voltammetry) [15], or this electrochemical detection can be associated with reversed-phase HPLC separation [16].

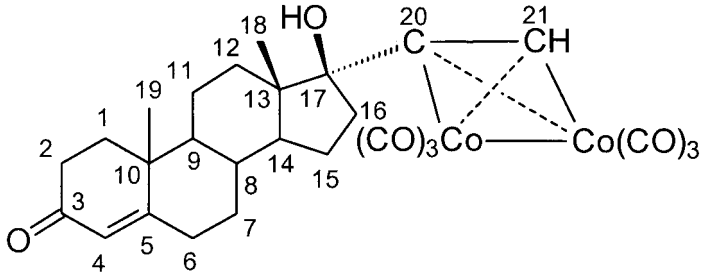

Fig. 1. *Structure of the [Co(CO)$_6$(ET)] complex*

2. Results and Discussion. – 2.1. *Synthesis and Spectroscopic Characterization*. The synthesis of [$Co_2(CO)_6$(ET)] was achieved simply by reacting [$Co_2(CO)_8$] and 17α-ethynyltestosterone (ET) at room temperature in an organic solvent. Characterization of [$Co_2(CO)_6$(ET)] was performed by comparing the perturbation induced by the

$Co_2(CO)_6$ moiety on the ^{1}H- and ^{13}C-NMR spectra of ET with those induced by the same moiety on 17α-ethynylestradiol.

In particular, the ^{1}H-NMR spectrum ($CDCl_3$) of [$Co_2(CO)_6$(ET)] shows a *s* at 6.11 ppm (C≡C–H) that is noticeably shifted (2.56 → 6.11 ppm) with respect to the spectrum of free ET, due to coordination to the $Co_2(CO)_6$ moiety. Similarly, in the ^{13}C-NMR ($CDCl_3$), the effect of the $Co_2(CO)_6$ group on the chemical shift of the acetylenic C-atoms C(20) and C(21) of ET is similar to that observed for the corresponding 17α-ethynylestradiol derivative (see *Table 1*).

Table 1. *Comparison of Selected ^{13}C-NMR Chemical Shifts* [ppm] *of [$Co_2(CO)_6$(ET)] and [$Co_2(CO)_6$(EE)].* ET = 17α-Ethynyltestosterone, EE = 17α-ethynylestradiol.

	ET[a])	[$Co_2(CO)_6$(ET)][a])	EE[a])	[$Co_2(CO)_6$(EE)][b])
C(20)	79.5	103.6	80.0	103.7
C(21)	74.8	73.6	74.9	73.5

[a]) Experimental data. [b]) [17].

The typical carbonyl stretching frequency pattern ($\tilde{\nu}$(CO) = 2092*m*, 2053v*s*, 2027*s*, 2021(sh)) confirmed the presence of a $Co_2(CO)_6$ fragment coordinated to a C≡C bond with an idealized C_{2v} geometry. The DCI-MS of [$Co_2(CO)_6$(ET)] exhibited fragments at *m*/*z* 656 (48%, $[M + C_4H_9]^+$, 600 (100%, $[M + H]^+$), and 582 (28%, $[M - OH]^+$), the latter signal indicating a moderate stabilization of the corresponding C(17) carbocation. We verified the purity of [$Co_2(CO)_6$(ET)] by means of HPLC (*RP-18* column, MeCN, flow rate 1 ml/min), a t_R of 4.50 min being recorded for [$Co_2(CO)_6$(ET)]; free ET, present only in traces, gave a very small signal at t_R 3.15 min.

2.2. *X-Ray-Diffraction Structure.* There are six molecules in the asymmetric portion of the unit cell (*Table 2*), four of which (*A*–*D*) present the same conformation as the one depicted in *Fig. 2* (molecule *A*). In this conformation, the Co–Co, Co–C, and C–C distances are in the ranges usually found for the [$Co_2(CO)_6$(alkyne)] derivatives [18]. The two different molecular conformations present in the crystal, *A*–*D* and *E* and *F*, are different with respect to rotations of the organometallic moiety (see differences in the torsion angle O(2)–C(17)–C(20)–C(21) in *Table 2*). This most likely results from some packing effects since there are no particularly strong intermolecular interactions in the crystal lattice.

Molecule *F* is the same as *E* except for the presence of some disorder such that the ethynyl bond in the $Co_2(CO)_6$ (C≡CH) unit in some of those molecules is longer, *i.e.*, more ethyne-like, but this disorder fraction (C(21*F*) → C(21*X*), where *X* refers to the *F*′ unit) amounts to *ca.* 8% (see *Table 2*, units *F* and *F*′).

2.3. IC_{50} *and Relative Binding Affinity* (*RBA*) *of [$Co_2(CO)_6$(ET)] for the Androgen Receptor* (AR). The IC_{50} and the relative-binding-affinity (*RBA*) value of [$Co_2(CO)_6$(ET)] for the AR were measured by a competitive method with the recently available recombinant AR expressed in *E. coli* (*PanVera LLC*, Madison, WI, USA) as the receptor source. The IC_{50} value found for [$Co_2(CO)_6$(ET)] was 1.45 μM (mean of 3 experiments), giving a *RBA* value of 0.5% for [$Co_2(CO)_6$(ET)] with a *RBA* value of DHT set by definition at 100%. This result shows that [$Co_2(CO)_6$(ET)] is still recognized by the AR even if, due to the high value of IC_{50}, the *RBA* value can be considered moderate.

Table 2. *Selected Bond Lengths* [A], *Bond Angles [°], and Torsion Angles [°] in the Co_2C_2 Core of the Six Units Found in the [$Co_2(CO)_6(ET)$] Crystal Structure.* For numbering, see *Fig. 2.*

	Unit *A*	Unit *B*	Unit *C*	Unit *D*	Unit *E*	Unit *F* (92%)	Unit *F'* (8%)
Bond lengths:							
Co(1)–Co(2)	2.461(2)	2.457(2)	2.454(2)	2.475(2)	2.445(2)	2.443(2)	–
C(20)–C(21)	1.296(1)	1.285(1)	1.261(1)	1.271(1)	1.275(1)	1.21(2)	1.38(2)
C(20)–Co(1)	1.954(9)	1.997(9)	1.956(1)	1.877(1)	1.953(1)	1.959(9)	–
C(20)–Co(2)	1.977(9)	1.963(9)	1.968(1)	1.955(1)	1.989(9)	1.978(9)	–
C(21)–Co(1)	1.897(1)	1.909(8)	1.917(9)	1.933(8)	1.901(8)	1.98(2)	1.83(2)
C(21)–Co(2)	1.881(1)	1.900(8)	1.925(9)	1.940(9)	1.933(8)	2.02(2)	1.81(2)
Bond angles:							
C(21)–C(20)–C(17)	145.4(9)	143.9(9)	144.1(9)	137.0(1)	149.0(1)	140.7(1)	155.2(1)
O(2)–C(17)–C(20)	106.3(7)	104.6(7)	105.5(7)	109.2(7)	107.0(7)	106.9(7)	–
Torsion angles:							
O(2)–C(17)–C(20)–C(21)	– 53.9(2)	– 69.1(2)	– 61.8(2)	– 57.8(2)	– 166.3(2)	– 160.7(2)	– 156(3)

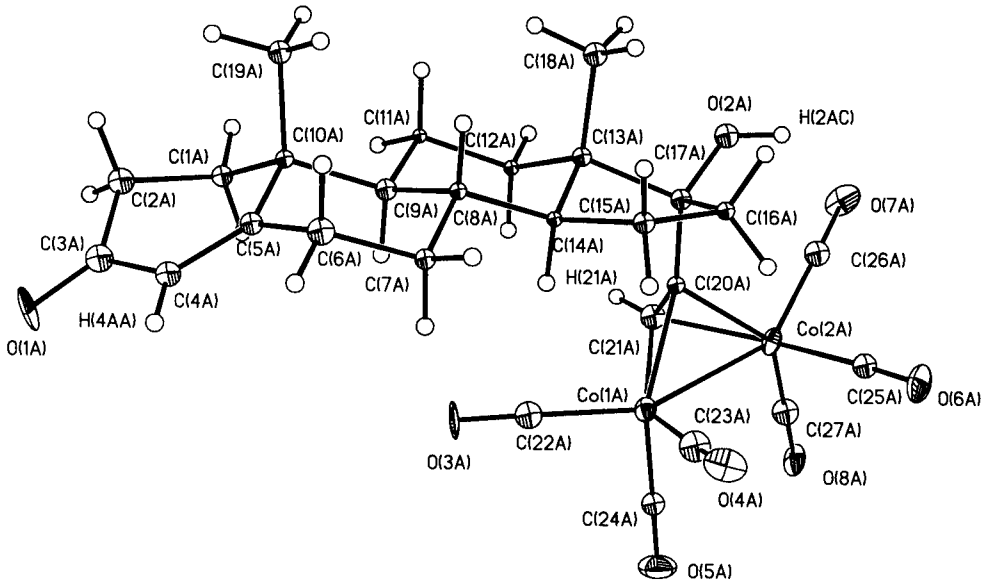

Fig. 2. *Molecular plot of [$Co(CO)_6(ET)$] with the atom numbering.* Thermal ellipsoids are drawn at the 50% probability level.

2.4. *Androgen Receptor [$Co_2(CO)_6(ET)$] Interaction.* The moderate RBA value found for [$Co_2(CO)_6$(ET)] and the recent availability of the AR structure with one molecule of DHT bound at the active site (AR · DHT) [19], prompted us to check qualitatively whether [$Co_2(CO)_6$(ET)] could fit the cavity of the AR.

The following calculations were performed with the HyperChem package: first an MM+ optimization of the X-ray structure of [$Co_2(CO)_6$(ET)] (unit *A*), to which the organometallic fragment $Co_2(CO)_6(C{\equiv}C)$ was attached, was undertaken. This process did not significantly change the geometry. The organometallic fragment Co_2-

$(CO)_6(C{=}CH)$ from the initially optimized structure was then linked to C(17) of the DHT moiety of the published structure of AR·DHT, to obtain the starting AR·$Co_2(CO)_6$(DHT)] model. During the next step (rigid docking), the whole [$Co_2(CO)_6$(DHT)] molecule was free to move inside the cavity to find the lowest potential. The [$Co_2(CO)_6$(DHT)] was then optimized, while the protein geometry was kept fixed to the AR·DHT published structure [19].

The optimization rearrangement forced two of the CO groups to be unusually bent. One Co–C–O bond deviates from the linearity seen in the sawhorse geometry, to 154° due to the interaction with the carbonyl group of Leu-30, the distance between the two O-atoms being only 1.86 Å. These results, at this qualitative level, clearly indicate that the cavity is not big enough to incorporate the entire [$Co_2(CO)_6$(alkyne)] moiety.

Furthermore, we computed with the MM+ force field the single-point energy (*SPE*) of AR·DHT (published structure) [19], and of DHT and AR (from the published structures) [19]. The binding energy *BE*(DHT) of the natural adduct was calculated from *Eqn. 1*. The low negative binding value (– 32.5 kcal/mol) is consistent with the natural reversible interaction between hormone and receptor.

$$BE(\mathrm{DHT}) = SPE(\mathrm{AR}\cdot\mathrm{DHT}) - [SPE(\mathrm{AR}) + SPE(\mathrm{DHT})] = -32.5\ \mathrm{kcal/mol} \quad (1)$$

An analogous calculation was performed on the corresponding [$Co_2(CO)_6$(DHT)] model. Single-point energies of AR·[$Co_2(CO)_6$(DHT)], AR, and [$Co_2(CO)_6$(DHT)] were used in *Eqn. 2* to compute the *BE*(model) for the model.

$$\begin{aligned} BE(\mathrm{model}) &= SPE(\mathrm{AR}\cdot[\mathrm{Co_2(CO)_6(DHT)}]) - \{SPE(\mathrm{AR}) + SPE([\mathrm{Co_2(CO)_6(DHT)}]\} \\ &= +247\ \mathrm{kcal/mol} \end{aligned} \quad (2)$$

We are aware that these calculated binding energies might be biased by the approximations made on modelling such a big and complex system that also contains transition metals. Nevertheless, the abnormally high positive value of the binding energy *BE*(model) confirms a nonbonding condition in the organometallic hormone model.

Further points that should be noted: looking at the structure of the AR, it immediately became clear that the hormone in the cavity is completely surrounded by amino acid residues, so that there is no free pathway for the hormone to get out (and in). This suggests that a stricter approach should not be based on a simple host-guest interaction, but should consider the active role of the tertiary structure of the AR, which can lead to the assembling of the receptor around the hormone derivative itself.

3. Conclusions. – The incorporation at the 17α position of 17α-ethynyltestosterone of a moderately bulky organometallic tag leads to a drastic drop in the affinity of the organometallic steroid for its specific receptor. This result, totally in contrast with the acceptable *RBA* values found for the organometallic derivatives of 17α-ethynylestradiol, is indicated by the very low *RBA* value and by an energy-optimized simulation of the insertion of the bioorganometallic derivative in the AR pocket.

Experimental Part

1. *General.* Reagents were purchased from *Sigma-Aldrich* and used as received. FT-IR Spectra: *Bruker Equinox-55*; matched air-free cells with NaCl windows, solvent CH_2Cl_2. NMR Spectra: *Jeol EX-400*; deuterated solvent as an internal lock; chemical shifts δ with respect to $SiMe_4$ (= 0.00 ppm). DCI-MS: *Finnigan-MAT-95Q* instrument with both magnetic and electrostatic analysers; the reagent gas (isobutane at 50 Pa) was initially ionized by electron impact (EI), yielding the stable *tert*-butyl carbenium ion; ionization of the analyte (*M*) by proton transfer from the carbocation generated the quasi-molecular ion $[M+H]^+$ of lower energy than M^+ generated by conventional EI and, therefore, $[M+H]^+$ underwent less fragmentation.

2. *Hexacarbonyl{μ-[(20,21-η:20,21-η)-(17α)-17-hydroxypregn-4-en-20-yn-3-one}dicobalt* ($[Co_2(CO)_6(ET)]$). To a soln. of (17α)-ethynyltestosterone (400 mg, 1.28 mmol) in CH_2Cl_2 (50 ml) solid $[Co_2(CO)_8]$ (450 mg, 1.32 mmol) was added, and the mixture was stirred at r.t. for 4 h under N_2. The reaction resulted in copious emission of CO, and the soln. color gradually changed from yellow-brown to dark-red. The progress of the reaction was monitored by IR (in particular: decrease of the 1866/1857 cm^{-1} bands of $Co_2(CO)_8$ [20] and increase of the highest-energy 'totally symmetric' [21] band at 2092 cm^{-1}). The reaction mixture was then separated by chromatography (short silica-gel column). After elution with petroleum ether until the yellow band of unreacted $[Co_2(CO)_8]$ completely disappeared, CH_2Cl_2/acetone 95:5 was used to collect a dark-red band. Evaporation and drying of this soln. under vacuum gave crude, solid $[Co_2(CO)_6(ET)]$ (500 mg, 90%).

This method essentially corresponds to published prep. procedures [22].

3. *Crystal-Structure Analysis:* Deep-red crystals of $[Co_2(CO)_6(ET)]$ suitable for the X-ray analysis were collected by dissolving the crude dark-red powder in warm hexane/CH_2Cl_2 9:1 (*v*/*v*), and letting this soln. slowly cool to r.t. under N_2. Crystallization occurred while the soln. was maintained at 4°. A suitable crystal of $[Co_2(CO)_6(ET)]$ was coated with *Paratone N* oil, suspended in a small fiber loop and placed in a cooled N_2 gas stream at 100 K on a *Bruker D8-SMART-APEX-CCD* sealed-tube diffractometer with graphite monochromated MoK_α (0.71073 Å) radiation. Data were measured by a series of combinations of ϕ and ω scans with 10-s frame exposures and 0.3° frame widths. Data collection, indexing, and initial cell refinements were all carried out with SMART [23] software. Frame integration and final cell refinements were done with SAINT [24] software. The final cell parameters were determined from least-squares refinement on 9999 reflections. The SADABS [25] program was used to carry out absorption corrections. The structure was solved by direct methods and difference *Fourier* techniques (SHELXTL, V5.10) [26]. H-Atoms were placed in their expected chemical positions with the HFIX command and were included in the final cycles of least squares with isotropic U_{ij}s by using a riding model. The C–H distances were fixed at 0.93 Å (aromatic and amide), 0.98 Å (methine), 0.97 Å (methylene), or 0.96 Å (methyl). All non-H-atoms were refined anisotropically. Scattering factors and anomalous dispersion corrections were taken from the 'International Tables for X-ray Crystallography' [27]. Structure solution, refinement, graphics, and generation of publication materials were performed with SHELXTL, V5.10, software. Additional details of data collection and structure refinement are given in *Table 3*.

4. *Receptor Binding Affinity of [$Co_2(CO)_6(ET)$] for the Androgen Receptor* (AR). Stock solns. ($1 \cdot 10^{-3}$ M) of $[Co_2(CO)_6(ET)]$ and DHT were prepared in DMSO and kept cold in the dark. Recombinant AR (ligand-binding domain, thioredoxin fusion, recombinant rat) expressed in *E. coli* purchased from *PanVera LLC*, Madison, USA, was used as the source of receptor and diluted in *Tris* buffer (50 mM, pH 7.5, 0.80M NaCl, 10% glycerol, 2 mM DTT (dithiothreitol), 0.1% BSA (bovine serum albumine)). Aliquots (200 μl) containing 1.47 pmol per tube were incubated in polypropylene tubes for 3 h at 0° with [1,2-^{3}H]dihydrotestosterone ($2 \cdot 10^{-9}$ M, specific activity 1.55 TBq $mmol^{-1}$, *NEN Life Science Products*, Boston) in the presence of nine concentrations of the competitor $[Co_2(CO)_6(ET)]$ or nonradioactive DHT. At the end of the incubation period, the free and bound fractions of [^{3}H]DHT were separated by protamine sulfate precipitate (1 mg/ml in H_2O) as described in [28]. The percentage reduction in binding of [^{3}H]DHT (*Y*) was calculated by the logit transformation of $Y(\text{logit}\,Y = \text{Ln}(Y/1-Y))$ *vs.* the log of the mass of $[Co_2(CO)_6(ET)]$. The IC_{50} of a compound is the concentration required to displace 50% of the bound [^{3}H]DHT. The *RBA* value of a compound is equal to: $100 \cdot IC_{50}$ DHT/IC_{50} compound. Consequently, the *RBA* value of DHT is by definition equal to 100%.

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Table 3. *Crystal Data and Structure Refinement for [$Co_2(CO)_6(ET)$]*

Empirical formula	$C_{27}H_{28}Co_2O_8$	
M_r	598.35	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	$P2(1)$	
Unit-cell dimensions	$a = 24.660(2)$ Å	$\alpha = 90°$
	$b = 12.919(1)$ Å	$\beta = 108.651(2)°$
	$c = 26.357(2)$ Å	$\gamma = 90°$
Volume	7956(1) Å^3	
Z	12	
Density (calculated)	1.499 Mg/m^3	
Absorption coefficient	1.298 mm^{-1}	
$F(000)$	3696	
Crystal size	$0.25 \times 0.20 \times 0.03$ mm^3	
θ Range for data collection	1.58–27.50°	
Index ranges	$-32 \le h \le 32$, $-16 \le k \le 16$, $-34 \le l \le 34$	
Reflections collected	104825	
Independent reflections	36516 ($R(\text{int}) = 0.1769$)	
Completeness to $\theta = 27.50°$	99.9%	
Absorption correction	Empirical	
Max. and min. transmission	1.00000 and 0.674253	
Refinement method	Full-matrix least-squares on F^2	
Data, restraints, parameters	36516, 1, 1159	
Goodness-of-fit on F^2	0.931	
Final R indices ($I > 2$ (I))	$R_1 = 0.0947$, $wR_2 = 0.1265$	
R indices (all data)	$R_1 = 0.1744$, $wR_2 = 0.1473$	
Absolute structure parameter	0.046(14)	
Largest diff. peak and hole	1.117 and -0.974 e·Å^{-3}	

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