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Mimesis of the Biotin Mediated Carboxyl Transfer Reactions

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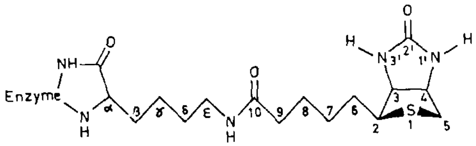
The lithio derivative of *N*-methylethyleneurea (or thiourea), a model for the isourea form of biotin, is capable of causing the rearrangement of 1-methyl-4-methylene-3,1-benzoxazin-2-one to 4-hydroxy-1-methyl-2-quinolinone. In a number of respects this reaction mimicks the carboxylation of biotin on nitrogen and the subsequent carboxyl transfer to a carbon atom bonded to a carbonyl group.

INTRODUCTION AND BACKGROUND

The prosthetic group biotin is found ubiquitously as a carbon dioxide transferring agent in carboxylations, decarboxylations, and transcarboxylations (*1*). A major present difficulty for chemists is to reconcile the structure of this coenzyme with the reactions it catalyzes (*2*). When viewed in conjunction with the striking advances that have been made in understanding the organic chemical aspects of the reactivity of, for example, NAD(P)H, tetrahydrofolic acid, thiamine pyrophosphate, pyridoxal phosphate, and vitamin B12, it becomes clear that biotin has been remarkably lacking in providing knowledge of the chemical principles underlying carbon dioxide transfer reactions.

There is a compelling reason to believe that the ureido ring of biotin plays a pivotal role in these various carboxylations. On the basis of trapping experiments carried out by Lynen and collaborators, the assumption has generally been made that the 1′-nitrogen of the ureido ring, which is sterically more accessible than the 3′-nitrogen, is carboxylated (*3*). These experiments were done with a carboxylase (β-methylcrotonyl-CoA-carboxylase), which implies the need of the ATP/Mg$^{2\oplus}$ system as an energy source for the reaction between carbon dioxide or bicarbonate and biotin. This nitrogen-bound carboxyl group is then transferred at a later stage to a carbon or nitrogen atom bonded



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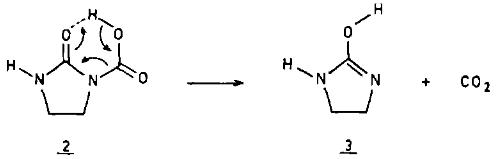
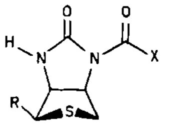
to a carbonyl group. There is very convincing experimental evidence that the carboxylation of biotin and the subsequent transfer of the biotin-bound carbon dioxide occur, in fact, at different sites on the enzyme. A popular model is that in which the biotin molecule acts as a carbon dioxide shuttle, moving back and forth between the two active sites by virtue of the conformational flexibility present in the long "tail" of biotin, which is lengthened by attachment to the ε-amino group of a flexible lysyl residue of the enzyme (**1**) (*4*).

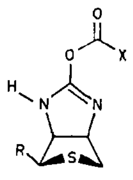
Using ethyleneurea (2-imidazolidinone) as a surrogate for biotin, Caplow demonstrated some years ago that the ureido group is a very poor nucleophile; indeed the only significant reaction of allophanic acids (*N*-carboxylated ureas) is decarboxylation under acidic conditions, probably via a six-membered transition state (**2**), leading initially to ethylene isourea (**3**) (*5*).



A resolution of the paradox of inert models but highly reactive biological systems was offered by Bruice and Hegarty, who suggested that the carboxylation of biotin occurs in fact not on the 1′-nitrogen (**4a**) but on the 2′-oxygen position[2] (see **4b**) (*6*).



4a



4b

The isolation by Lynen and co-workers of 1′-methoxycarbonylbiotin in a trapping experiment using diazomethane, a result considered one of the strongest arguments for identifying 1′-*N*-carboxybiotin as the actual carbon dioxide carrier, is not inconsistent with this proposal. There is a well-established precedent for the spontaneous and rapid rearrangement of either a carboxyl group or a methoxycarbonyl group from oxygen to nitrogen (**4b**→**4a**); organic chemists have long been aware of, and have studied in detail, this type of rearrangement (*7*).

The attractiveness of the Bruice and Hegarty proposal lay in the fact that biotin would be carboxylated on its most nucleophilic site, the urea oxygen, and that on carboxyl transfer the leaving group would be biotin in the low energy urea form, closely analogous to urea as leaving group in the Khorana peptide synthesis by way of carbodiimides, in which an isoimide is also the intermediate (*8*). The mechanism of catalysis would also bear resemblance to the mode of functioning of tetramethylurea (TMU) as a solvent and

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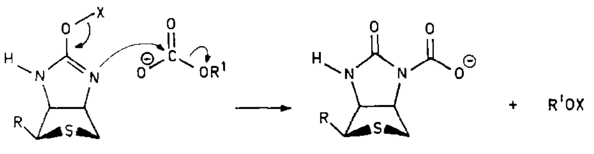
[2] *O*-carboxybiotin was first proposed by Lynen as an alternative mechanism (*3a*) but this early suggestion has not been cited since 1961 (*1b*, *3b*).

catalyst in acylation reactions (*9*). Finally, the postulation of *O*-carboxybiotin as the true intermediate provided a convincing explanation for the observation that chemically synthesized 1′-*N*-carboxylated biotins were always found to be inactive when added to enzymic systems (*3b*, *c*, *10*). In view of the many arguments for the validity of the Bruice and Hegarty proposal, it came as a considerable shock when Guchhait and others provided very convincing evidence that in fact 1′-*N*-carboxybiotin must be a true biochemical intermediate. The elegant experiments of this group seem to eliminate 2′-*O*-carboxybiotin as an intermediate, and return us to the original problem of finding the ways by which biotin and carboxybiotin are activated (*11*).

We considered, following an earlier suggestion of Hegarty, Bruice, and Benkovic (*12*), the possibility that biotin reacts in its high energy isourea form by means of the generalized scheme of Eq. (1).



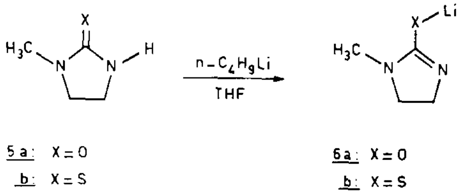
(1)

Activation is achieved by attack of an electrophile, X, followed by deprotonation. X could be a proton or a metal ion[3] from the active site of the carboxylase, or a phosphoryl group[4] delivered by ATP. R′ can stand for a proton or a phosphoryl group. A scheme in which X and R′ represent the same phosphoryl group is also attractive.

We wish to report a catalyzed isomerization that incorporates aspects of this idea and which, moreover, mimics an essential aspect of the second half-reaction, namely the transfer of the carboxyl group from biotin nitrogen to an acceptor molecule.

RESULTS

N-Methylethyleneurea and the analogous thiourea (**5a**, **b**) are lithiated (**6a**, **b**) on treatment with *n*-butyllithium in dry tetrahydrofuran (THF).



(2)

The *N*-methyl derivatives were used to block one acidic hydrogen and to improve the solubility of the models in THF. The infrared spectra of **6a** and **b** measured in 0.1–0.01 *M*

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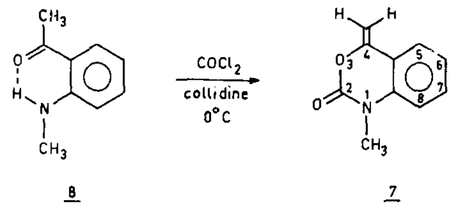
[3] The possibility of attack of an ureido anion upon a carboxyl anion was shown also by Hegarty and Bruice in 1969 (*13*).

[4] An *O*-phosphobiotin mechanism was first proposed by Lynen (*3a*, *1a*). A model for such a mechanism, in which a phosphonyl group, via a methylene bridge, is attached to a ureido nitrogen has recently been published (*14*). For a hypothetical mechanism in which biotin is acetylated by the carboxyl acceptor, acyl-CoA, see Kohn (*15*).

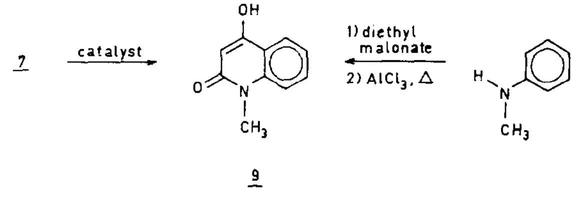
solutions in THF indicated that the lithium derivatives are certainly predominantly in the isourea form. The strong carbonyl absorption of **5a** at 1720 cm^{-1} changes in **6a** to a strong imine absorption at 1600 cm^{-1}. No indication could be obtained for the presence of a carbonyl absorption. In an analogous fashion, the moderate thiocarbonyl absorption of **5b** at 1560 cm^{-1} disappears on lithiation and is replaced by a strong imine absorption at 1605 cm^{-1}. A ^{13}C-nmr spectrum of **6b** taken under the same conditions is also indicative of only one tautomer of **6b** being present.

As substrate for catalyzed isomerization we designed a methylene analog of *N*-methylisatoic anhydride (1-methyl-4-methylene-3,1-benzoxazin-2-one) (**7**). This material, not previously described in the literature, was obtained by reaction of *N*-methyl-*ortho*-aminoacetophenone (**8**) at 0°C in toluene with phosgene in the presence of excess collidine (Eq. (3)).



(3)

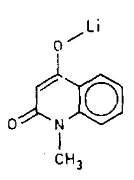
In theory one can imagine the existence of a biotin-like catalyst capable of transferring the nitrogen-bound carboxyl group in **7** (C_2) to the carbon of the exocyclic methylene group (carboxy acceptor), leading to 4-hydroxy-1-methyl-2-quinolinone (**9**) shown in Eq. (4). An unambiguous synthesis of **9** was carried out as shown on the right-hand side of Eq. (4) (*26*).



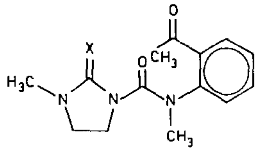
(4)

No trace of isomerization of **7** was observed in solvents including tetrahydrofuran (THF), acetone, acetonitrile, dimethylsulfoxide, or toluene with or without added potential catalysts like LiI, $R_4N^{\oplus}I^{\ominus}$, pyridines, or BF_3OEt_2. At high temperature (up to 180°C in closed vessels) the only reaction observed was darkening of the solution and slow polymerization. The ureas **5a, b** were likewise completely ineffective with or without added pyridine or collidine. It was satisfying therefore to find that **7**, on treatment with either **6a** or **6b** in dry THF[5] at reflux for 15 min, produced a light yellow precipitate that turned out to be the lithium salt of **9** (**9′**), formed in 47–49% yield (multiple experiments carried out). The balance of the material was apparently a polymer of undetermined structure (ca. 30% yield), a 10–15% yield of unreacted **7**, and 5–10% of a compound to which structure **10a, b** was tentatively assigned.

[5] At first sight dry THF may seem a peculiar solvent for a biomimetic reaction, but there is evidence that the biotin mediated reactions take place in a cleft of the enzyme, where water as solvent is possibly absent (*16*).

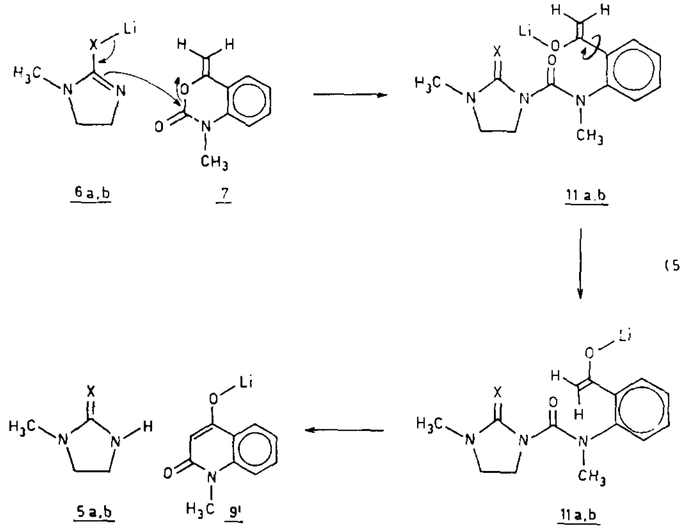


9'



10a,b

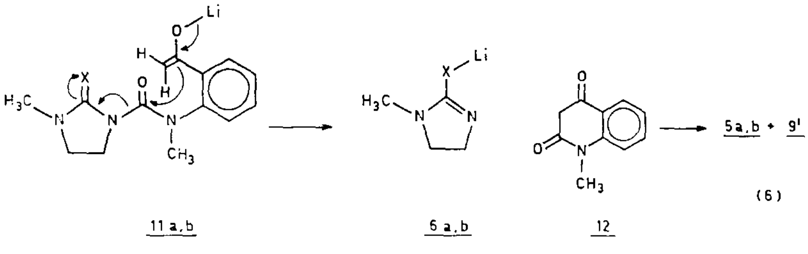
A mechanism appropriate for the 7→9′ transformation is that shown in Eq. (5).



(5)

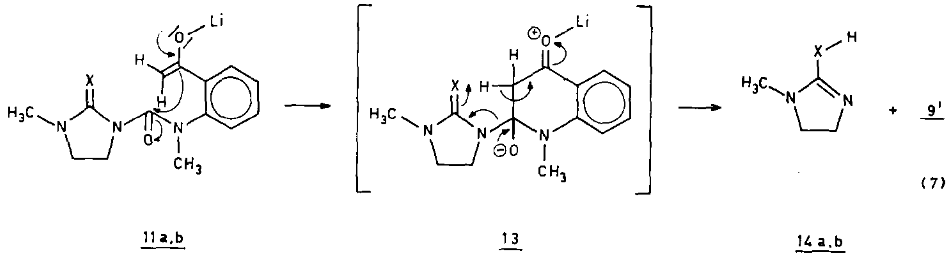
The basicity of **6a, b** is expected to be considerably greater than that of **9′**, leading to production of the ethylene (thio)urea, **5a, b**. This proton is therefore available for the neutralization of the intermediate **11a, b** to produce **10a, b** (tentatively identified). The course of the reaction is not that simple, as seen from the low yield of (presumed) **10**; the important point, however, is that under the conditions of the experiment, the yield of **9′** cannot exceed 50%. This is in good agreement with the observed yield of the rearrangement.

The exact course of the ring-closure reaction (the parallel of the carboxyl transfer step) is also uncertain, especially regarding the nature of the leaving group. Two types of neutral leaving groups are conceivable: first, **6a, b**, leading to **12**, a tautomer of **9** (Eq. (6));



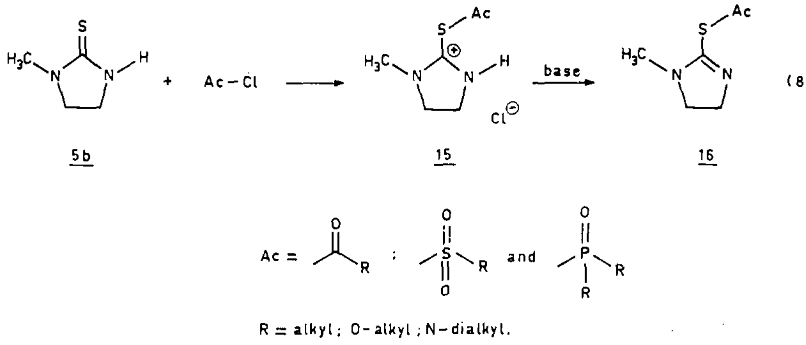
(6)

and second, **14a**, **b**, the isourea forms of **5a**, **b**, which can arise via a structure like **13**, leading directly to **9′** (Eq. (7)).



For the mechanism in Eq. (6), the more familiar one to an organic chemist, one has to invoke several intermolecular lithium transfers, whereas the mechanism in Eq. (7), which goes completely intramolecularly, bears more resemblance to the mechanism proposed for the enzyme catalyzed carboxyl transfer (*17*).

Attempts to prepare stable isoimides of the ethylene(thio)urea system along the lines of Eq. (8) have not yet been successful, which is not surprising in view of the known tendency of such compounds to rearrange intra- or intermolecularly to the more stable *N*-acyl derivatives.

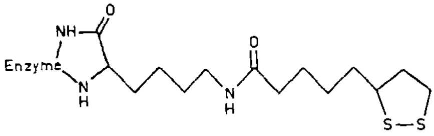


We wished to examine whether such compounds, being analogous to *O*-phosphobiotin (see Eq. (1), $X = PO_3^{2\ominus}$) could also be effective as catalysts in the transformation of **7** into **9**. The instability of such isoimides precludes, unfortunately, any such investigation. In the oxygen series only 2-alkyloxy- and 2-aryloxy-2-imidazolines are known; the *O*-acyl compounds, although doubtlessly generated through deprotonation of appropriate salts, have only a short lifetime and even escape detection. It has been shown, however, that 2-methoxy-2-imidazoline is a powerful nucleophile, capable of attacking the carbonyl group of a variety of substrates (*12*). This is consistent with our observation that the lithiated isourea form has also good nucleophilic capacities. Of equal interest, although the point is unproved, is that the *N*-substituted isourea (**6a**, **b** or **14a**, **b**) can be eliminated by a carbon nucleophile, which in this case has the advantage of proximity to enhance its nucleophilicity. We believe these observations may indeed provide a strong hint regarding the mechanism of catalysis by biotin.

CONCLUSION AND SPECULATION

The above chemistry based on model systems corroborates, in our view, a growing conviction that the function of biotin is *not* like that of the other coenzymes, namely, to act as a transferring agent of an *activated* synthon in biological systems (cf. "active acetyl, methyl, methylene", and so on). If the carboxyl group of carboxy biotin has an "abnormally" high transfer potential, and it is indeed found that enzyme-bound carboxybiotin is a labile entity (*1b*), then this must be attributed entirely to the enzyme portion of the holoenzyme. Biotin-like molecules seem to have no detectable catalytic power in carboxylations unless they have been activated first to the high energy tautomer, which is appreciably more nucleophilic. It is therefore misleading to use terms like "activated carbon dioxide" or "biotin catalyzed carboxylation" for the biotin-mediated reactions. Biotin seems to act only as a carbon dioxide shuttle, linking two different active sites together, but does not contribute itself to the lowering of the activation energy, as the other group transferring coenzymes do. This explains why biotin, unlike the other coenzymes, has not been very cooperative in model studies.

Peculiarly enough, biotin is, together with the structurally related enzyme bound lipoic acid **17**, also an eccentric among the coenzymes in other respects. Biotin and lipoic acid bear no relationship to a nucleotide.



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All other coenzymes are nucleotides, or at least contain a cyclic nitrogenous base which could be derived from a nucleotide. This fact coincides with some recent speculations about the origin of life (*18*). The earliest stages of self-reproducing systems are in this theory nucleic acid-like molecules coding for nucleic acid enzymes, proteins not yet being important as such. Present-day coenzymes and t-RNAs, which can be considered as very large coenzymes, are then the vestiges from that polynucleotide enzyme era and still have some of the catalytic power for group transfer reactions (*19*). Ribosomal RNA may very well be a polynucleotide that even today acts as an enzyme, and other polynucleotide enzymes might still be operative, especially in DNA replication (*20*). Biotin and lipoic acid have no tendency to act as a coenzyme when not attached to a polypeptide enzyme. They are only transferring agents in multi-enzyme systems. Could all this indicate that biotin and lipoic acid as prosthetic groups are essentially late evolutionary developments?

EXPERIMENTAL

Melting points were determined with a Reichert melting point microscope and are uncorrected. The pmr spectra were taken on a Varian A-60 spectrometer with TMS as internal standard. The ^{13}C-nmr spectra were taken on a Varian XL-100 instrument.

The ir spectra were recorded with an Unicam SP200 spectrometer. Elemental analyses were carried out by the Micro-Analytic Department of the Chemical Laboratory of the University of Groningen.

N-Methylethyleneurea (**5a**)

We followed the method of Fischer for ethyleneurea (*21*). The procedures of Frick and others (*22*) and of Hall (*23*) were, in our hands, not satisfying. Equimolar quantities of *N*-methylethylenediamine and diethylcarbonate were mixed and heated in a Carius tube to 180°C for 8 hr. After cooling, white crystals appeared. The yield after crystallization from toluene was 90%. Before use it was sublimated twice at 1 mm Hg/120°C. The melting point is no criterium for purity. Sharp melting points varying from 68–118°C have been reported (*22, 23*); polymorphism is involved. After sublimation one sees slow melting from 70–116°C. In the end new crystals appear. The compound is hygroscopic. Ir: $\nu_{c=o}$ 1720 cm^{-1}. ^{13}C-nmr: δ 163.9, 47.9, 38.2, and 30.5 ppm[6].

N-Methylethylenethiourea (**5b**)

We followed the method of McKay and Kreling (*24*). After crystallization and sublimation, the yield was 80%. Mp 131–132°C. ^{13}C-nmr: δ 186.9, 51.2, 41.2, and 33.7.

O-Lithio-N-methylethyleneisourea (**6a**) *and S-lithio-N-methylethyleneisothiourea* (**6b**)

These were prepared by slowly adding a 10% excess of *n*-butyllithium (15% in hexane) to a solution of 1 mmol of **5a, b** in 10 ml of carefully dried THF (twice distilled from $LiAlH_4$; only freshly distilled THF was used). When not exposed to air or daylight the solutions were stable for several days. Ir (taken with 0.1 and 0.01 *M* solutions in THF) **6a**: $\nu_{c=n}$ 1600 cm^{-1}; **6b**: $\nu_{c=n}$ 1605 cm^{-1}. ^{13}C-nmr **6b**: 185.8, 53.5, 50.0 and 35.6 ppm[6]. A ^{13}C-nmr of **6a** could not be obtained under the same conditions, owing to the lower solubility of **6a** in dry THF.

1-Methyl-4-methylene-3,1-benzoxazin-2-one (**7**)

To a solution of 3.73 g of (**8**) (25 mmol) and 10.55 g of collidine (3.5 equiv) in 500 ml of dry toluene at 0°C an excess (ca. 5 equiv) of $COCl_2$ gas was slowly (ca. 2 bubbles/sec) added over a total period of 1.5 hr while vigorously stirring. The yellow solution slowly turned colorless, and a white precipitate (collidine–HCl and collidine–$COCl_2$) appeared. After further stirring for 1 hr at 0°C the precipitate was removed with a glass filter (P3) and destroyed. The excess $COCl_2$ in solution was removed by distillation, and the freshly formed precipitate was filtered off after 30 min standing at 0°C. After removal of the solvent a greasy yellow substance remained. The crude product was washed thoroughly with 50 ml of pentane for 30 min, powdered, washed another time with 50 ml of pentane, and dried, giving 3.95 g (90%) of pale yellow crystals, with mp 64.5–65.5°C. Recrystallization from ether or ether/pentane did not raise the melting point further. Exposed to air and daylight, the compound darkens slowly (several days). Pmr ($CDCl_3$): δ 7.6–6.9 (*m*, 4*H*); 4.94 (*d*, 1*H*, $J = 3$Hz); 4.83 (*d*; 1*H*; $J = 3$Hz); 3.42 (*s*, 3*H*). Ir: $\nu_{c=o}$ 1730; $\nu_{c=c}$ 1640 cm^{-1}. *Anal.* Calcd: C, 68.56; H, 5.17; N, 7.99. Found: C, 68.24; H, 5.10; N, 7.91.

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[6] Proton decoupled, taken with a 0.1 *M* solution in THF in a 1-cm tube with external deuterium lock (D_2O), using THF as standard: δ (TMS) 25.8 and 67.9 ppm.

N-Methyl-ortho-aminoacetophenone (**8**)

Synthesized following Kempter et al. (*25*).

4-Hydroxy-1-methyl-2-quinolinone (**9**)

Synthesized following Ziegler et al. (*26*).

Transformation of (**7**) *into* (**9′**)

To a solution of 1 mmol of **6a, b** in 5 ml of dry THF 175.2 mg of **7** in 5 ml of THF was added. After 15 min of reflux the yellow precipitate was separated by filtration, washed twice with pentane, and dried; giving a yield of 87 mg (48%). The precipitate was dissolved in 3 ml of water and acidified with 0.1 *N* HCl. Pale yellow crystals of **9** were separated by filtration and dried by washing with dry ether. Identification by mp, ir, and nmr. Mp 264–265.5°C; ir: $\nu_{c=o}$ 1630 cm^{-1}; pmr (DMSO-*d6*): δ 11.50 (broad, 1*H*); 7.20–8.05 (*m*, 4*H*); 5.92 (*s*, 1*H*); 3.55 (*s*, 3*H*).

The mother liquor of the reaction mixture was neutralized with HCl gas, evaporated, and extracted with deuterated chloroform. A glassy material remained (ca. 30%), soluble only in DMSO (pmr only broad absorptions). The nmr and ir of the deuterochloroform extract revealed the presence of **5a, b** (60%), **7** (10–15%), and 5–10% of a compound which could well be **11a, b**. In the ir, both compounds absorb at 1700 cm^{-1} (aryl ketone) and 1720 cm^{-1} (urea carboxyl). The pmr gives, besides the methyl absorption of the *N*-methylethylene(thio)urea group, methyl absorptions of equal height at δ 2.48 and 3.28 ppm for **11a**, and δ 2.53 and 3.29 ppm for **11b**. However, this structure can be assigned only tentatively, because **11a** and **b** could not be isolated from the reaction mixture.

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