

Process Development Implications of Biotin Production Scale-Up

Aleksander Warm,* Andrew B. Naughton, and Elie A. Saikali

Lonza AG, CH-3930, Visp, Switzerland

Abstract:

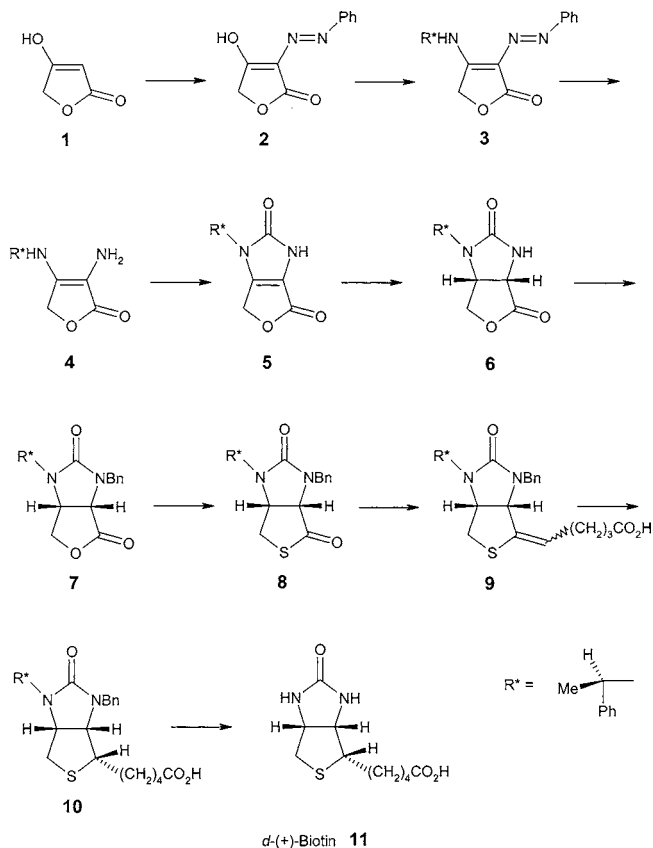
Careful planning and construction of suitable in-process tests and isolation of new impurities observed led to quick, mechanistic-based assessments of problems seen in two steps of Biotin production scale-up and allowed for rapid changes to be made to resolve the issues. The quench of Grignard reaction mixtures with deuterium-based materials led to increased understanding of one of the main pathways for yield loss in a Grignard reaction contained with the Biotin process.

Introduction

Vitamin H, more commonly known as biotin, is an essential part of the metabolic cycle causing catalytic fixation of carbon dioxide (carboxylation) in the biosynthesis of organic molecules. The current world demand for synthetically produced biotin is about 35 metric tons per year, of which about 85% is needed for the feed market, and about 15% for pharmaceutical and cosmetic applications. There are a handful of producers of biotin, of which Hoffmann-La Roche is clearly the largest. In 1997 an excellent review by De Clerq of the synthetic development on the biotin system appeared.¹ Special notice was made of the contributions of the scientists at Hoffmann-La Roche, and the author also mentioned the scarcity of detailed information available on the actual commercial production of biotin. In 1988, Lonza AG patented² an exclusive route for the production of biotin. The route started from a Lonza building block, tetronic acid **1**, and was very robust during initial small-scale piloting (Scheme 1).³ The final product was of excellent over-all quality.

At the heart of the route were four essential steps, the diastereoselective hydrogenation of olefin **5**, the potassium thioacetate (KSAc) mediated lactone-to-thiol-lactone conversion producing intermediate **8**, the di-Grignard-mediated conversion of **8** to **9**, and the methanesulfonic acid deprotection of **10** to produce D-(+)-biotin **11**. Although the thioacetate conversion⁴ and the di-Grignard reaction⁵ had precedence in the biotin literature, there was, as mentioned by De Clerq, a lack of detailed information about the commercial production of these two steps. The methanesulfonic acid deprotection was a considerable improvement

Scheme 1



over the previous HBr-based deprotection, in particular regarding the amount of halogen in the waste stream. However, despite successful development of the industrial synthesis, and the subsequent introduction in 1994⁶ of an even more diastereoselective hydrogenation to intermediate **6**, Lonza decided in 1998 to leave the biotin business.

In 1993 we were called upon to transfer steps 6–10 of the piloted process to larger 6- and 11-m³ reactors. Due to the substantial increase in scale as compared to that in the original piloting and the relatively high price per kilogram of finished biotin, it was decided that much stricter process controls would be put into place during the scale-up than those used during the piloting. In steps 6 (*N*-benzyl), 9 (hydrogenation), and 10 (deprotection) these tests proved useful for completion of reaction, but they provided little further insight into the process itself and did not indicate any differences to the laboratory or pilot processes. Unlike these three steps, however, the in-process analytics in steps 7 (thiolactonization) and 8 (Grignard reaction) and subse-

* To whom correspondence should be addressed. Telephone: ++41-27-948-6522. E-mail: aleksander.warm@lonzagroup.com.

(1) DeClerq, P. J. *Chem. Rev.* **1997**, *97*, 1755.

(2) McGarrity, J.; Tenud, L.; Meul, T. Eur. Pat. Appl. EP 0270076 A1, 1988; McGarrity, J.; Tenud, L. Eur. Pat. Appl. EP 0273270 A1, 1988.

(3) In large part, this meant 600-L reactors.

(4) Gerecke, M.; Zimmermann, J.-P.; Aschwanden, W. *Helv. Chim. Acta* **1970**, *53*, 991.

(5) Isaka, I.; Kubo, K.; Takashima, M.; Murakami, M. *Yakugaku Zasshi* **1968**, *88*, 964.

(6) Eyer, M.; Merrill, R. PCT Int. Appl. WO 94/24137 A1, 1994; McGarrity, J.; Spindler, F.; Fuchs, R.; Eyer, M. Eur. Pat. Appl. EP 0624587 A2, 1994.

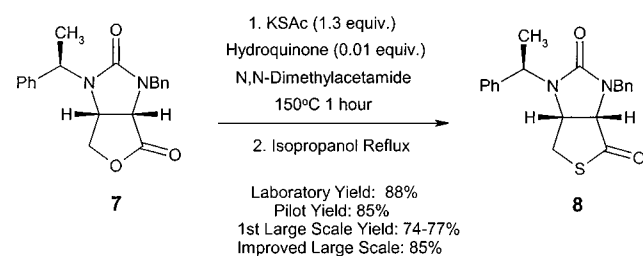
quent isolation and identification of previously unobserved impurities were key to providing quick answers to scale-up issues and led to better understanding of the process as a whole. The changes introduced in the processes as a result of these understandings led to an improvement in overall yield and quality during scale-up, and finally, to the robust production desired. It is hoped that these examples will prove both illustrative and interesting concerning how scale-up problems in batch manufacturing can be approached. It is also hoped that the information provided will contribute to filling the gap in the literature on commercial biotin production, as noted by De Clerq.

Process Details, Problems, and Solutions

To properly describe the results, some attention must now be paid to the details of both steps.

Intermediate 8: KSAC Thiolactonization. In detail, the chemistry of thiolactone **8** was worked out during initial piloting and is shown in Scheme 2. A detailed laboratory procedure is in the Experimental Section.

Scheme 2



During initial investigations two critical parameters were identified. The first was the quality of the potassium thioacetate (KSAC). However, the quality of KSAC was not defined in a measurable way since no adequate analytical method was in place. Certainly it was known that the storage stability of KSAC was limited. The material stored for longer periods was darker colored and prone to give lower yields in the process, resulting in the recommendation that all KSAC be freshly produced. The second critical parameter, seemingly more related to product quality, was the length of time that the reaction mixture would be held at the maximum reaction temperature of 150 °C. The highest product assays were obtained with precisely 1 h at 150 °C, but there was evidence that up to 4 h heat-up time until 150 °C was acceptable. The KSAC decomposition pathway and kinetics at elevated temperatures were not known, but iodometric titrations of its solutions in *N,N*-dimethylacetamide (DMAA) under nitrogen showed that as much as 43% of the KSAC decomposed after a 1 h hold at 150 °C either with or without hydroquinone as an antioxidant. Thus, it seemed important to ensure the shortest possible heat-up time and to keep the maximum temperature hold time to the minimum necessary allowing for the desired conversion.⁷

To ensure the highest quality KSAC it was decided to run the thiolactone (**8**) production process with in-house freshly prepared material. Despite this measure, and excellent quality

(7) Subsequent experiments showed that by adding KSAC at 140 °C the process yield could be raised 5%.

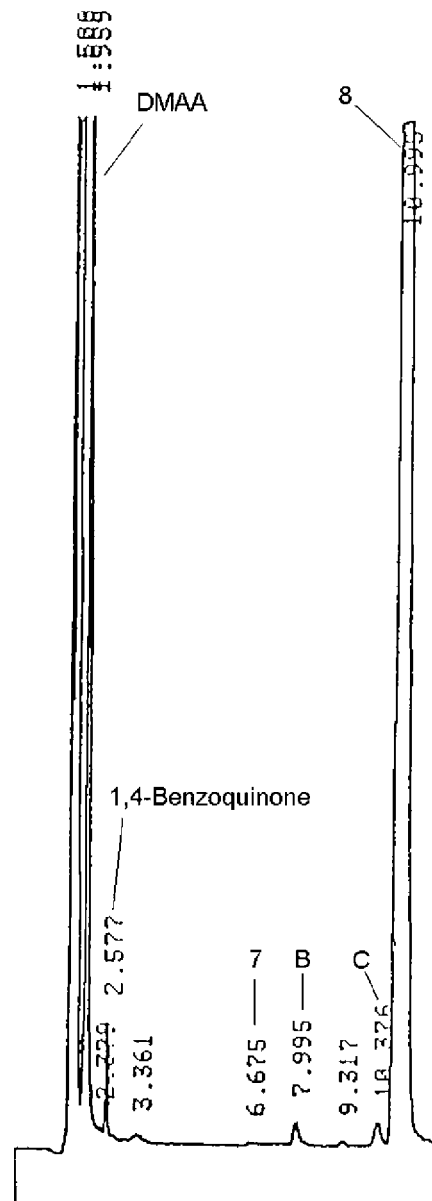
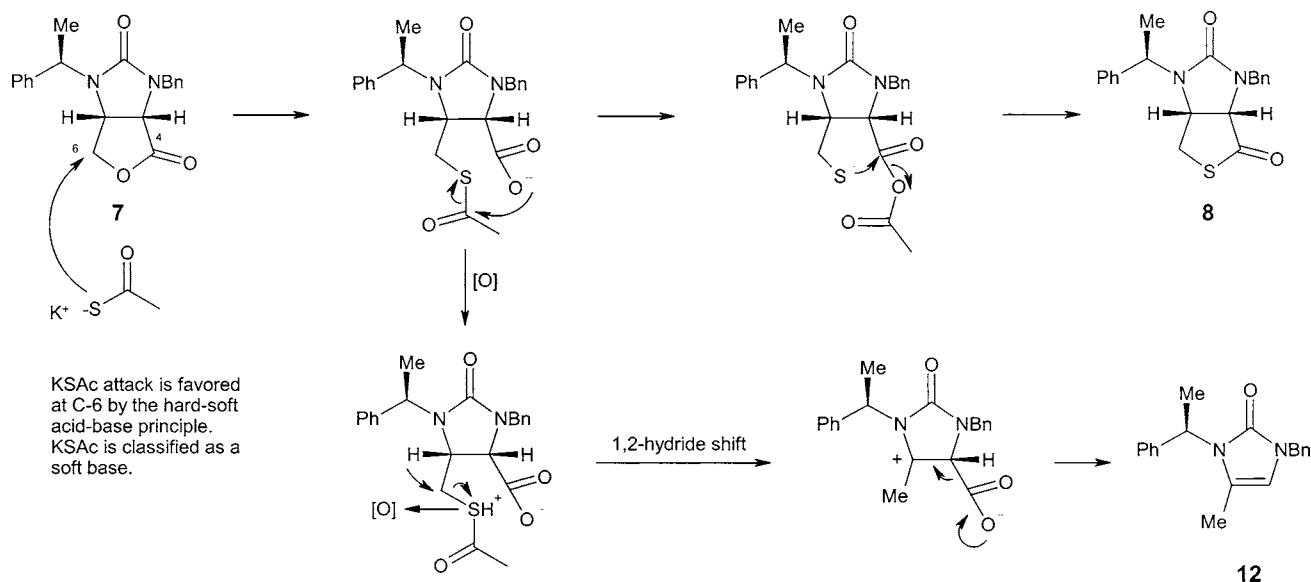


Figure 1.

of the starting material **7**, the yields of **8** were 10% lower than in the pilot campaign and unusually dark-colored product was obtained in most batches (dark brown instead of the desired light beige). The laboratory use-tests of these colored batches showed that even though they behaved normally in the Grignard step, the resulting intermediate **9** required more catalyst in hydrogenation to the intermediate **10**—a sign, perhaps, that some sulfur contamination was getting through? As a result of this observation a charcoal pretreatment had to be added to the first production batches of **8** going into the Grignard reaction.

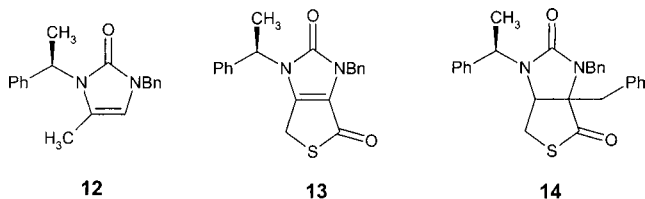
A reversed-phase HPLC-based in-process test was developed to replace the less exact TLC method used in piloting. A normal chromatogram taken at the end of the 1-h hold on a total reaction mixture is shown in Figure 1. A limit of 0.2% (a/a) was set for the remaining **7**, and this measure was passed for all batches. Also seen in the HPLC were two unknown peaks at an earlier retention time as compared to that for **8**. As these compounds were unknown

Scheme 3



to us, they were labeled as **B** and **C**. It was also postulated that **B** was either quite polar or smaller than **8** as there was 7 times as much of it to be found in the DMAA aqueous liquor as there was **8**. Both **B** and **C** were to be found in the 2-propanol slurry liquor (see Experimental Section).

As all of the KSAc had been freshly produced, after the first production campaign attention was refocused on the second critical parameter, namely, the time-temperature profile. However, extensive experiments with the heat-up time showed it was even less critical than previously believed.⁸ Seeking clues to why the reaction was different in piloting, efforts were undertaken to identify the darkly colored side products. Thus, the impurities **B**, **C**, and the previously unobserved **D** were isolated from the 2-propanol slurry liquor by column chromatography and identified as structures **12**, **13**, and **14**, respectively. The identity of compound **C** was confirmed by comparison with an authentic sample from an earlier synthetic route. Compound **14** is likely a very small impurity that was concentrated enough to be isolated.



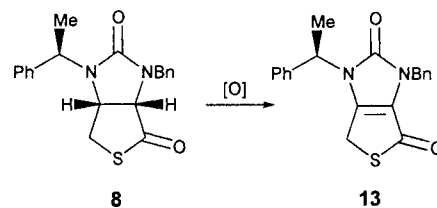
Compound **12** is the major impurity. The related dibenzyl-protected compound is also known⁹ and served as support for the structure of **12**. None of these impurities was darkly colored, as had been expected. The origins of compounds **12** and **13** are postulated as oxidative side products of the reaction. The formation of **12** is more simply explained by attack of thioacetate at the soft acid C-6 of **7**, but not easily by attack at C-4 (Scheme 3).

(8) Lab experiments would become clear at ca. 135 °C, and beige crystals would fall out at ca. 150 °C. Very likely the reaction only occurs at a meaningful rate over 135 °C.

(9) Isaka, I.; Kubo, K.; Takashima, M.; Murakami, M. *Yakugaku Zasshi* **1968**, *88*, 1062.

Compound **13** very likely emerges from **8** itself (Scheme 4). The skeletal origins of **14** are not clear, but it seems quite probable that its precursor lactone originated in the previous step.

Scheme 4



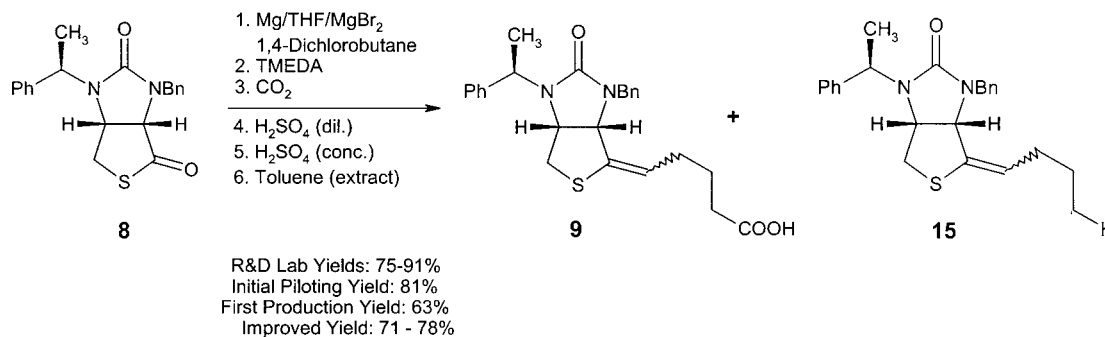
Although, in retrospect, their formations seem obvious, **12** and **13** were unexpected by-products from the reaction. Their direct importance can be questioned, since their structures do not suggest that they could form the basis of a hydrogenation catalyst poison two steps hence. However, they indicate the presence of oxygen in the reaction mixture.

Although we were aware of the danger of oxidative degradation of KSAc, for which reason the reaction was carried out under inert atmosphere and in the presence of the antioxidant hydroquinone, the allowable oxygen concentration was not specified. Due to the off-gas removal system the reactor was under a slight negative pressure during the reaction, causing some normally acceptable atmospheric seepage. Unless special precautions are taken, plant nitrogen may also contain some level of oxygen; normally, all that is guaranteed is that oxygen will be below the level to support combustion. Samples of nitrogen can be purchased with measured levels of oxygen, and upon running the reaction under an increasing content of oxygen the colored batches and higher levels of **12** and **13** can be reproduced (Table 1).

A level of 1% oxygen in the reaction headspace seemed to be a technically acceptable compromise. Interestingly, it also did appear as if hydroquinone could be removed from the process based on other experiments, as it did not play any observable role.

Table 1. Influence of oxygen level and heat-up time on 8

	atmosphere	HPLC in-process test (1 h, area %)			8	
		12	13	8	color	% yield
1	air (0.5 h heat up)	2.7	2.0	95.3	dark brown	73.7
2	N ₂ with 5.12% O ₂ (0.5 h heat up)	1.8	1.5	96.7	light brown	82.1
3	N ₂ with 1% O ₂ (3 h heat up)	0.8	0.9	98.3	beige	87.2
4	N ₂ (0.5 h heat up, 3 ppm O ₂)	0.4	0.4	99.2	beige	88.9
5	argon (0.5 h heat up, <3 ppm O ₂)	0.5	0.4	99.1	beige	87.3

Scheme 5

Of the factors leading to oxygen in the reactor, the negative draw and reactor leaks were considered the most problematic in comparison to the pilot plant. A quickly introduced provisional solution to the problem was found in adding a restrictor valve to the off-gas system and building a small positive pressure on the reactor. This removes the influence of leaks that add oxygen to the reactor (being, however, not acceptable as a long-term process change due to the danger of atmospheric pollution). After this simple change, the yields returned to the high level of the pilot campaign, and the batches of **8** were all mostly light beige, only occasionally becoming sandy-colored in appearance—no further dark batches were obtained. After extensive laboratory testing of these new batches it was also possible to remove the charcoal pretreatment of **8**. Although the new impurities **12** and **13** were probably not the root cause of the problems associated with the colored batches, they are a symptom of the root cause, and their structures suggested a probable intrusion of oxygen into the system. Oxygen probably contributes to the breakdown of KSAC at high temperatures yielding sulfur-containing impurities that act as catalyst inactivator two steps later. The solution was therefore realized in quicker fashion through identification and explanation of the small impurities observed in the in-process test.

Intermediate 9: Di-Grignard Reaction. The chemistry of intermediate **9** was worked out during initial piloting and is shown in Scheme 5. A detailed laboratory procedure is in the Experimental Section.

Shown as a second product of the reaction in 10–15% yield is the butylene side-chain by-product **15**. This compound was known from the earliest lab experiments on this reaction, and since it is an oily side product removed to a level below 0.5% during crystallization, it was accepted as a yield loss, but of no consequence to the quality of **9**.

Preliminary experience showed that there were two highly critical parameters. During formation of the di-Grignard

reagent **16** (Scheme 6) the reaction had to be maintained at 30–35 °C to obtain the maximum conversion. The second critical parameter was that –20 °C to –30 °C had to be maintained during addition of the solution of **8** to **16** and then afterwards during the CO₂ addition forming the carboxylic acid group. This last factor was never tested in piloting, as the evidence was sufficiently strong from laboratory batches; large yield losses would result from being above this temperature although the mechanistic basis for this loss was never explained.

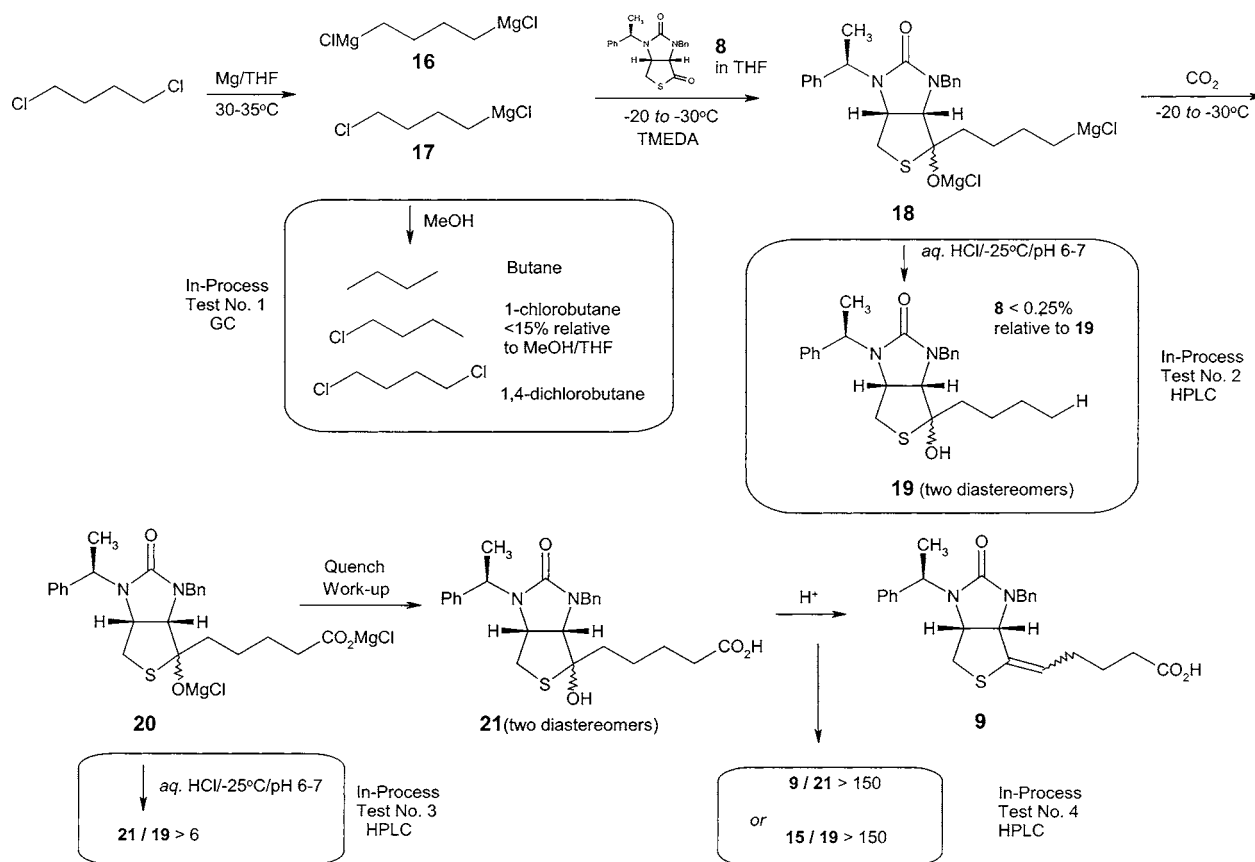
Due to the complexity of the process—there are three main reactions in this sequence, spanning five large reactors for each batch—in-process tests were devised for each step in the sequence and are detailed in Scheme 6.

The first in-process test was designed¹⁰ to assess the conversion of di-Grignard reagent formation by GC-analysis of a sample quenched with methanol. Although butane originating from the di-Grignard reagent was not quantifiable due to its volatility, an assessment of Grignard strength was indirectly possible by estimation of chlorobutane that emerged from methanol quench of the intermediate mono-Grignard reagent **17**. The formation of **17** itself was obviously always complete since the unreacted starting material 1,2-dichlorobutane was never observed. The coupling of thiolactone **8** with di-Grignard reagent produces a key intermediate, the adduct **18**. The conversion of this reaction was verified by a second in-process test performed on the sample quenched at controlled temperature and pH.

In the third in-process test the completion of the CO₂ addition was also judged from a carefully quenched sample. As will be discussed, laboratory results had shown that conversion of **18** to di-Mg-salt **20** could only rarely reach more than 85%, additional CO₂ added in an attempt to raise it being of only limited effectiveness. The last in-process test

(10) Implemented at piloting stage by Dr. D. Quarroz, Lonza AG.

Scheme 6



was designed to verify a seemingly trivial conversion, the acid-catalyzed dehydration of the tertiary alcohol. However, it needs to be remembered that before this final reaction several operations such as a Grignard quench, solvent exchange into toluene, a phase separation, and an azeotropic distillation were taking place. It was thus quite possible that too much water made it through the workup, causing the elimination reaction to fail. In such a failure it was necessary to remove the spurious water and re-run the elimination.

Setting limits to the tests 2 and 4 was fairly obvious, as the disappearance of starting materials was required. The Grignard test limit in test 1 was set on a series of representative batches produced in the laboratory where about 15 mol % mono-Grignard **17** remained unconverted but the final **9** was nonetheless of good quality. In plant practice the amount of **17** was always below 4.6%, very likely owing to the extended time of reaction in the larger vessel as opposed to the laboratory-scale vessel. The limits of test 3, the CO₂ addition, were more difficult to set, but proved to be the basis of a good deal of understanding about the course of the reaction and key to resolution of the eventual problems.

Figure 2 shows the results of sequential CO₂ in-process tests that were used to set the limits of the in-process test on a lab batch with increasing aliquots of CO₂ added. Next to the chromatogram is found the **21/19** ratio. As can be seen in the first two chromatograms, and again on the last, a shoulder peak was occasionally observable on the first eluting peak of **19** (*t*_R = 12.0 min). Since it was impossible to be sure whether this peak (labeled as impurity E) was always resolved, the area of the shoulder was counted with the peak

of **19**. Thus defined, the **21/19** ratio became a quantitative measure for the completion of the CO₂ addition.

A quench prior to the CO₂ addition results exclusively in the diastereomers of **19**, the E shoulder also being visible. A normal amount of CO₂ to be added was divided into six portions and added separately, each time letting the exotherm pass before taking a sample and adding the next portion. It was interesting to note that, although the first exotherm was quite pronounced, almost no **21** was produced. Although speculative, this is probably due to the excess of the Grignard reagent being quenched. Thereafter the **21/19** ratio continued to grow until all of the CO₂ had been added and CO₂ had been passed over the reaction for an additional hour.¹¹ A final ratio of 5.58 was obtained. It should be mentioned that the 1-h hold corresponded to an end to observable exothermic behavior. In a subsequent laboratory batch that finished its 1-h CO₂ hold with a ratio of 7.56 an additional 12 h of CO₂ only raised the ratio to 8.26, which corresponds to a calculated conversion increase of only 0.9%. On the basis of these experiments the in-process test 3 limit was set to **21/19** ratio > 6.

Although the first two in-process tests of the first large-scale batch showed better than expected results, the third test began to show a sharp contrast to the lab and pilot experience. As shown below in Figure 3, a **21/19** ratio of barely 3.66 was reached at the end of the hold time and two additional hours under CO₂. No further exothermic behavior was observed. Remarkably, what was earlier the impurity E

(11) CO₂ was added over the surface of the reaction. Other modes of CO₂ addition (solid, subsurface) were not found to be more efficient.

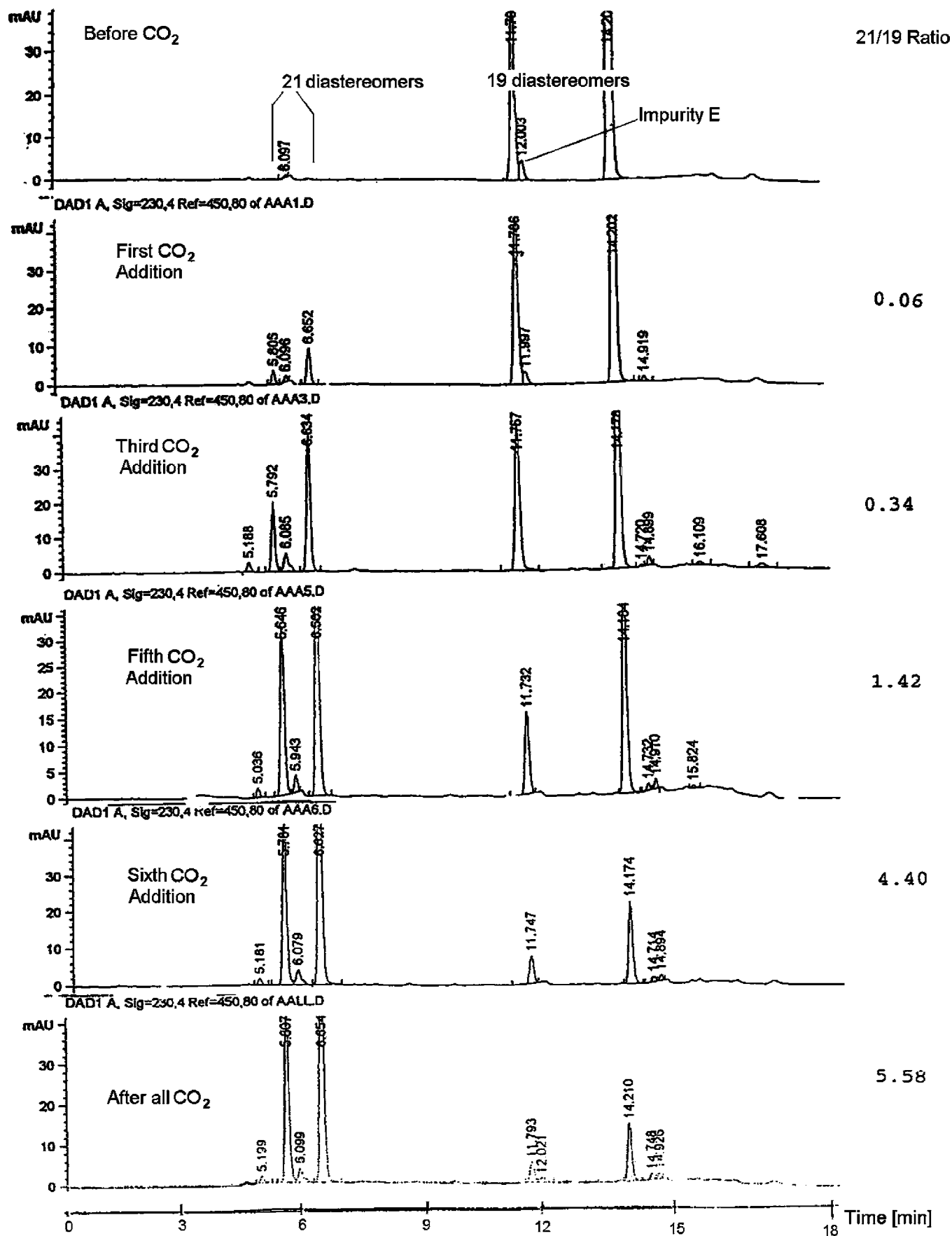


Figure 2.

shoulder had now taken up a major position and was larger than the first of the two diastereomers of 19.

At the water elimination step leading to 9 we were met with another surprise. As shown in Figure 4, the large-scale

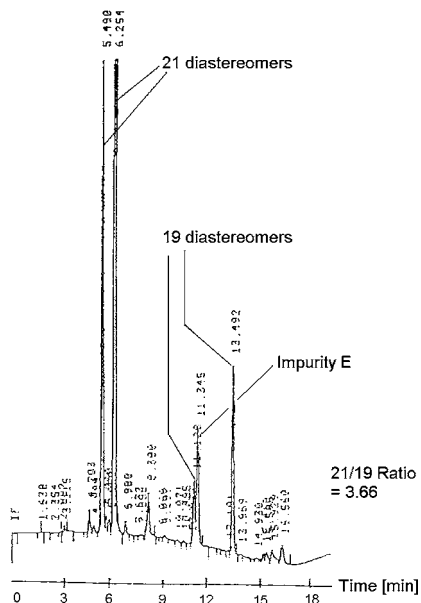


Figure 3.

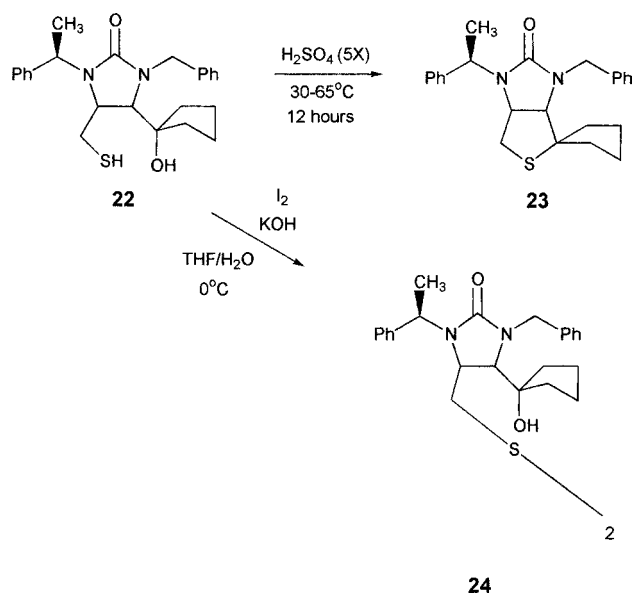
reaction in-process test (shown on the right) indicated a much less clean reaction than in the laboratory (left).

In laboratory reactions only very small amounts of polar by-products were observed in the region of **21**, and they did not interfere with assessment of whether the elimination reaction was complete. However, in the large-scale batches these polar by-products became so pronounced that it was impossible to assess the completion of the reaction except by examining the cleaner region around **19** and judging the decay of the second diastereomer of **19**.

Finally, the work-up procedure and crystallization were not very effective at removing the increased level of these polar by-products (Figure 5 right: HPLC of plant **9**, left: lab sample). Although it was proven that despite the lower purity of **9** an acceptable quality of the final product was achievable, the associated yield losses could not be tolerated. Thus, the major challenge for further development was to improve the selectivity of the particular steps of the process so that the yield would meet the initial goal.

The impurity **E** peak was the only real physical clue to the difference in chemistry between lab- and plant scale, and its isolation and identification were seen as a possible key to the problem. Fortunately, this task was made easier by recalling an abnormal instance of the depletion of **8** test (in-process test 2). In the normal instance of in-process test 2, the sample was quenched immediately with dilute acid and therefore was not allowed to warm over $-20\text{ }^{\circ}\text{C}$. In an abnormal lab instance, a sample was taken at $-20\text{ }^{\circ}\text{C}$, but was allowed to warm to room temperature over more than an hour before being quenched. In this test the impurity **E** peak was larger than each of the peaks of the **19**-diastereomers. This phenomenon was obviously analogous to what we observed in the early plant batches at the in-process 3 level (Figure 3). As a consequence, a lab reaction was carried out, and allowed to warm to room temperature for 1 h directly after addition of **8** before being quenched. This caused further conversion of the **18**-diastereomers into **E** that was then isolated and identified as a thiol **22** (Scheme 7).

Scheme 7



The assignment of **E** as a thiol **22** was originally based on the presence of a triplet at 1.39 ppm that exchanged with D_2O only very slowly (characteristic for thiols¹²). Mass spectral analysis was inconclusive and suggested that the compound under question could also be the dimeric disulfide **24**. The proposed structure **22** was then submitted to oxidative reaction conditions¹³ and was completely transformed into a new product. The FAB-MS of the oxidation product showed the expected calculated isotope pattern for the dimeric disulfide **24** and therefore the identities of both **22** and **24** were confirmed. The *N,N'*-dibenzyl thiol analogous to **22** can also be found in older biotin literature.⁵ Recall from above, the yield loss due to increased formation of the thiol **22** was only half of the problem. The other half was the increase in polar by-products that were poorly removed by the crystallization. To see if these by-products were, at least in part, emerging from **22**, it was treated with concentrated sulfuric acid in the same fashion that the alcohol elimination was carried out. It took 5 times the normal amount of acid and 11 h to get the reaction to complete, but in the end the decidedly nonpolar spirocyclic thioether **23** was obtained as the major product, with no sign of polar degradative by-products.

As before in the thiolactonization step, the identification of the impurity suggested the source of at least one problem. Even at the prescribed low temperature of below $-20\text{ }^{\circ}\text{C}$, the extended time of addition of **8** allowed for larger amounts of **22** to form. Laboratory and plant data were quickly arranged to consider the time of addition of **8**, and new experiments were carried out with precisely defined **8** addition times. Some of these experiments are presented in Table 2.

After the first plant results were available, a new criterion based on the information obtained from in-process test 4 was

(12) Silverstein, R. M.; Bassler, G. C. *Spectrometric Identification of Organic Compounds*, 2nd ed.; Wiley: New York, 1967; p 124.

(13) Vogel, A. I.; Tatchell, A. R.; Smith, P. W.; Rogers, V.; Hannaford, A. J.; Furniss, B. J. *Vogel's Textbook of Practical Organic Chemistry*, 4th ed.; Longman: New York, 1978; pp 585–587.

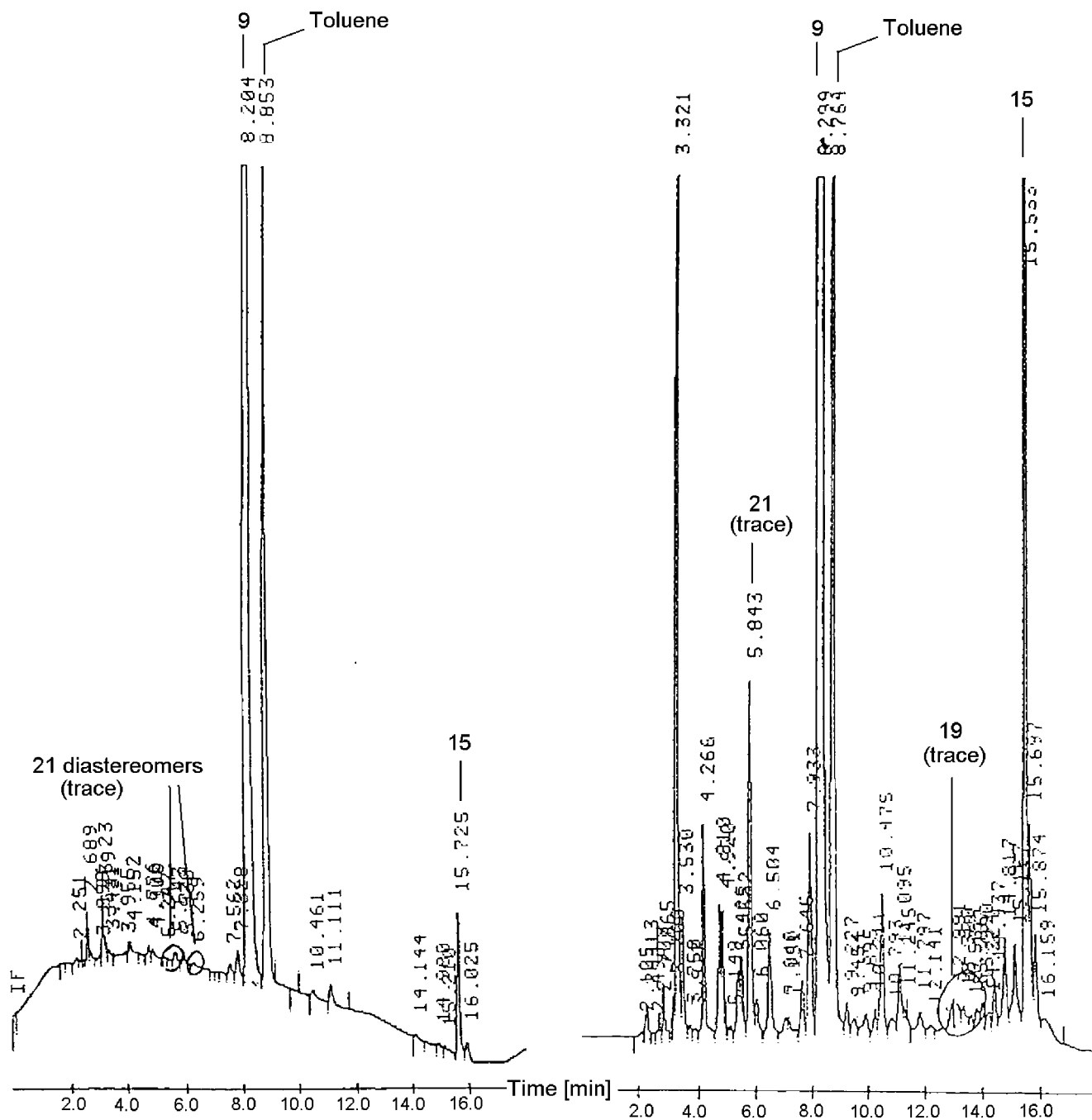


Figure 4.

defined—the area of the emerging peak of **9** was divided by the total area of the polar impurities below a certain retention time in the HPLC. This became a measure of the “cleanliness” of the batch going into the recrystallization. Higher numbers attested to “cleaner” mixtures that would likely crystallize to good product, whereas lower numbers would be more difficult to crystallize in high assay.

Experiments 1 and 2 showed typical laboratory and kilo lab results. Although the **21/19** ratio occasionally fell below the desired limit of 5.5, the ratio of **9** to polar by-products remained quite high, as did the corresponding final product assay. Experiment 3 is a representative early plant batch characterized by an extremely long addition time of **8**. As mentioned previously, the **21/19** ratio is clearly lower, and the polar by-products are greatly increased. Experiments 4–6

were carried out with exaggerated **8** addition times to reinvestigate this phenomenon in the laboratory once the thiol **22** had been identified. Although the **21/19** ratio was not always extremely accurate at predicting the success of the batch, the level of polar impurities rose reliably as addition time increased.

Even though the correlation with the **21/19** ratio was not absolute, the data suggested a strong enough correlation between the quality/yield of **9** and the **8** addition time to justify the installation of an external recirculation loop (shell-and-tube heat exchanger) to increase the heat-transfer capacity of the reactor system and cut the addition time of **8**. The loop was operated during the addition of **8** in that the reaction mass was recirculated through the cooled exchanger in addition to the cooling via reactor jacket. Experiments 7 and

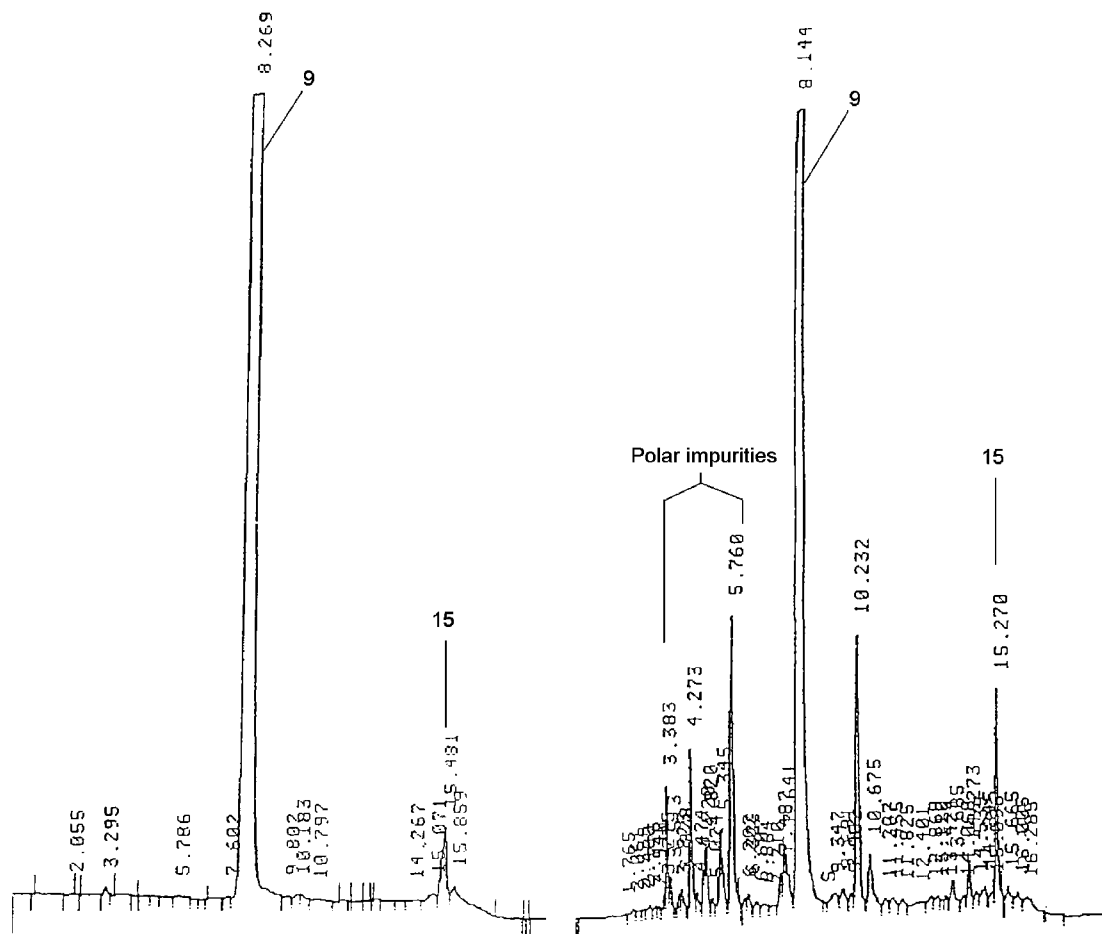


Figure 5.

Table 2. Influence of addition times of 8 on the Grignard reaction

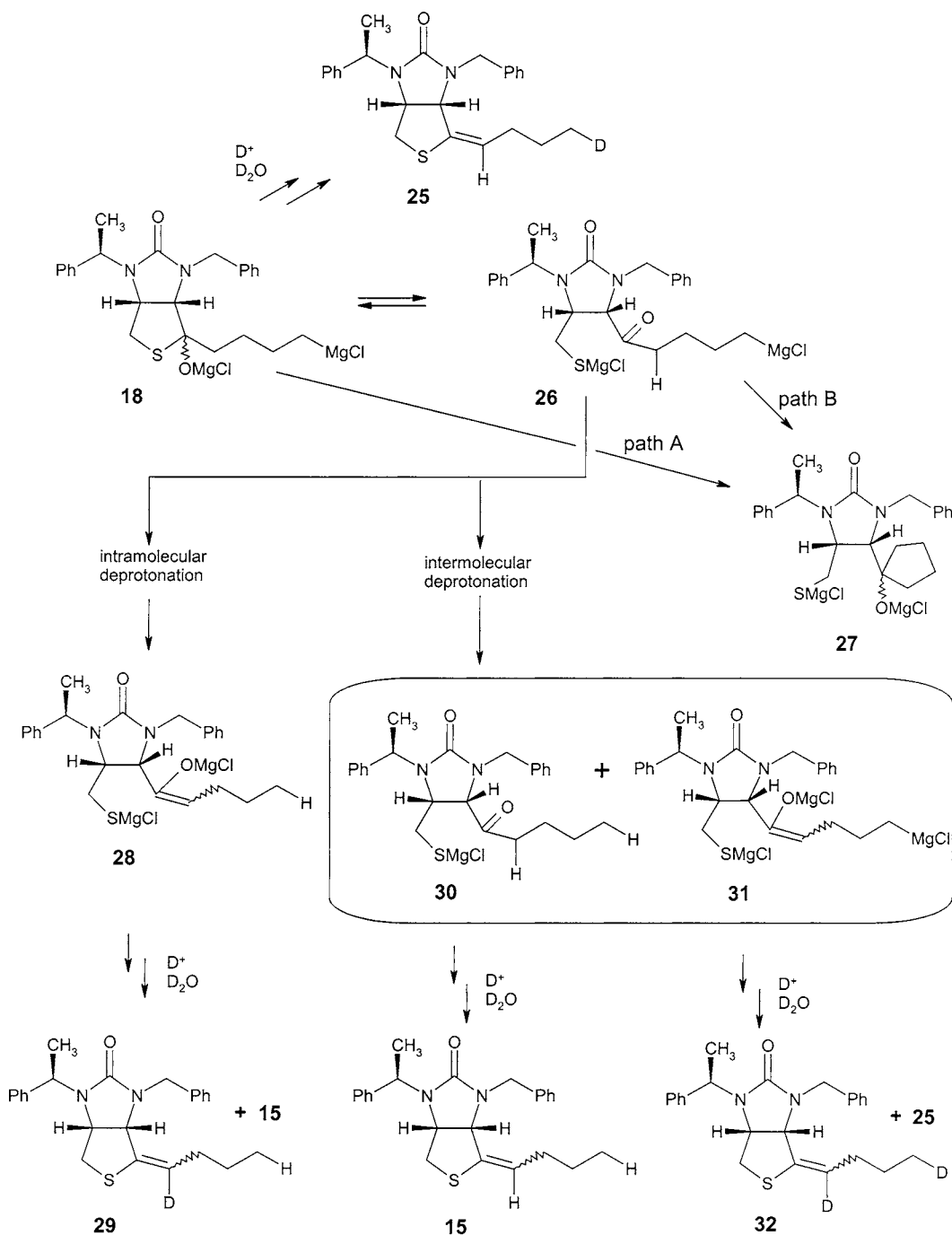
expt	location	8 addition time	21/19 ratio	9/polar products ratio	9 yield (%)	9 assay (%)
1	development lab	45 min	5.48	150.0	73.0	95.8
2	kilo lab	50 min	6.25	n.a. ¹⁴	79.7	93.7
3	production	11.3 h	3.55	10.8	67.4	89.1
4	development lab	2 h	5.87	159.4	80.1	95.8
5	development lab	5 h	4.28	29.0	69.4	94.0
6	development lab	10 h	3.51	12.3	60.1	79.0
Install Cooling Loop						
7	production	1.2 h	5.33	162.7	76.6	94.7
8	production	1.1 h	5.45	165.6	78.0	94.1

8 are plant batches carried out after the loop installation. The addition time of 8 was now normally less than 2 h using this new system. Although the 21/19 ratio numbers are clearly higher than those in early batches, they still show some irregularity in predicting yield and assay of final products. However, the ratio of 9 to polar by-products shows no such doubt, and the quality of all final products in this series matched that of the laboratory (compare Figure 5). After installation of the recirculation loop the product yield rose by an average of 9.2%, and the average product assay rose by 4.2%.

As mentioned before, by-product 15 was long accepted as the largest yield loss in 9. The reasons for its formation were not entirely clear. Although the water content normally determined in the starting materials, reagents, and solvents could account for as much as 3.5% of 15, it was also difficult

to accept that 10–15% of 15 could be formed by this or other traces of spurious moisture. More acceptable was the idea that after a certain amount of CO₂ had been added the reaction mixture (suspension) underwent a phase change and permitted no further CO₂ to reach the adduct 18. A reviewer points out that although phase changes are common in Grignard chemistry they are not usually perceived as the limiting parameter. Nevertheless, for most of the development effort this was the dominant hypothesis. However, the isolation of the thiol 22 added a new dimension to the possibilities that we sought to verify. The fact that a side product was isolated wherein the thieno ring had opened led to the suspicion that 22 is at least partially formed via equilibration of 18 with its opened keto-form 26 (Scheme 8). As a consequence, deprotonation and enolization of 26 by any highly basic Grignard agent might also be possible.

Scheme 8



A simple quench of the reaction mixture after addition of **8** with DCl in D₂O could lead, in principle, to three types of **15** with deuterium incorporation: **25**, **29**, and **32**, and to nondeuterated **15**. The mixtures of these species are further called **15(D)**.

In the instance where adduct **18** existed exclusively as a closed thieno-ring compound at $-20\text{ }^{\circ}\text{C}$ the di-Mg-salt **27** of thiol **22** would have to be formed by an intramolecular attack of Grignard moiety resulting in the opening of the thieno ring and formation of the cyclopentane ring (path A). Also in such a case **25** would be the only product of deuterium quench, and deuterium NMR would show no evidence of an olefinic deuterium. However, ²D NMR of **15(D)** in CCl₄¹⁵ isolated from a normal reaction mixture after

addition of **8** over 1 h and then quenched quickly into a solution of DCl in D₂O showed not only the methyl deuterium peak but also a smaller olefinic signal¹⁶ obviously belonging to molecules of **29**- or **32**-type. Approximate integration of the olefin signal shows it to be about 8% of the area under the methyl signal. ¹H NMR of this **15(D)** clearly shows the presence of the deuterium at the methyl position (integral and coupling pattern), but there was no noticeable distortion¹⁷

(14) This parameter was not identified/recorded at this early stage of development.

(15) Control spectra were made. Addition of CDCl₃ caused another peak to emerge, and biotin H quenched with H₂SO₄/H₂O showed no signals in ²D NMR.

(16) Slight chemical shift difference from ¹H NMR due to the fact that the spectra were taken in different solvents, CCl₄ for ²D NMR, CDCl₃ for ¹H NMR.

of the signal at the olefinic region so that it is likely the incorporation of deuterium at the olefin is quite low, as suggested by the deuterium integration. The ^2D NMR result strongly suggests that at least some part of adduct **18** is an open ring form **26** at $-20\text{ }^\circ\text{C}$ that also can give rise to thiol **22** via its di-Mg-salt **27** by intramolecular addition of Grignard moiety to the carbonyl (path B).

Unfortunately, while the deuterium quench prior to CO_2 does strongly suggest the existence of the ring-opened form of **18**, it does not help to differentiate the pathways leading to **15** (due to a predominant presence of **25** from adduct **18** in the quenched reaction mixture). By adding CO_2 to **18** in the normal way and then quenching with $\text{DCI/D}_2\text{O}$ we reasoned that one could obtain a clue to the major pathway to **15**. By doing so, we obtained after chromatographic separation **15(D)** that showed the same ^2D NMR spectrum as in the case before, *however the ^1H NMR showed no noticeable distortion in the terminal CH_3 signal.* This is taken to mean that, while there is a portion of **15(D)** which is monodeuterium-substituted ($-\text{CH}_2\text{D}$), it is small compared to the tris-proton-substituted variant ($-\text{CH}_3$). We believe this to mean that practically all of **15** that is formed originates in a process that occurs before aqueous quench. This would not be possible if the largest pathway to **15** was caused by a phase change in the mixture which prevented CO_2 from reaching the terminal Grignard reagent—in such an instance the largest portion of **15(D)** obtained with $\text{DCI/D}_2\text{O}$ quench would be deuterium-substituted at the terminal position, and one would clearly see the coupling pattern caused by the terminal methyl deuterium in ^1H NMR and a reduced methyl proton integral as in the preceding experiment. If we discount as small the probability of spurious moisture before quench as leading to most of **15**, this would leave the enolization process of **26** as the largest probable cause of formation of **15**.

Conclusions

Careful planning and construction of suitable in-process analytical tests and isolation of impurities seen therein led to quick, mechanistic-based assessments of problems seen in biotin production scale-up and allowed for rapid changes to be made to resolve the issues. Such changes would have been significantly delayed at much higher cost without an understanding of the reaction pathways brought about by those tests. Further investigations led to even more detailed understanding of the process and its traditionally accepted yield/loss avenues and provided leads to follow for further process improvements.

Experimental Section

General. In the following, unless otherwise stated, ^1H NMR spectra were recorded on a Varian XL 300 MHz NMR in CDCl_3 solution with a multinuclear probe that also acquired ^{13}C NMR spectra at 75 MHz. All chemical shift values are reported in ppm downfield from reference of tetramethylsilane, and coupling constants are reported in hertz

(Hz); for ^{13}C NMR the off-resonance decoupled split pattern is reported in parentheses. ^2D NMR spectra were recorded in CCl_4 on a Bruker 500 MHz NMR that was tuned to deuterium at 76.7 MHz. FTIR spectra were recorded on a Mattson FTIR and are reported in wavenumbers (cm^{-1}). Chemical ionization (CI) mass spectra were determined with a Fison Instrument VG 70-70 E spectrometer at 70 eV ionizing voltage using CH_4 for ionization. Fast atom bombardment (FAB) mass spectra were determined with a Vacuum Generators Micromass 7070E spectrometer at 6 kV acceleration voltage using argon for ionization in glycerine matrix. Mass to charge (m/z) is reported with (relative intensity) values in parentheses.

Procedures. (3aS,6aR)-1-[(R)-1-Phenylethyl]-3-benzyltetrahydro-1H-thieno[3,4-d]imidazol-2,4-dione (8). A mixture of **7** (100 g, 0.29 mol), hydroquinone (0.32 g, 0.0028 mol) and potassium thioacetate (44.08 g, 0.39 mol) in 130 mL of *N,N*-dimethylacetamide was stirred in a 500-mL double-walled glass reactor. The system was nitrogen-purged three times before being heated to $150\text{ }^\circ\text{C}$ under nitrogen for exactly 1 h. The suspension became a clear-brown solution at ca. $135\text{ }^\circ\text{C}$, and beige crystals were formed when the reaction reached ca. $150\text{ }^\circ\text{C}$. The suspension was cooled to $100\text{ }^\circ\text{C}$ followed by the addition of glacial acetic acid (1.77 mL, 0.03 mol). The suspension was cooled to $55\text{ }^\circ\text{C}$ over 30 min, and 432 mL of water was added. The suspension was stirred at $55\text{ }^\circ\text{C}$ for 1 h at room temperature. Filtration followed by a water wash (240 mL) afforded crude **8** (99.9 g, 0.28 mol) as a light-brown/beige solid material. The crude **8** was then dissolved in 2-propanol (675 mL) at reflux. This solution was then cooled to $0\text{--}5\text{ }^\circ\text{C}$ over a period of 3–4 h. The product was isolated by filtration and dried in a vacuum oven at $50\text{ }^\circ\text{C}$ until the loss on drying was $<0.5\%$. **8** was obtained as a beige solid (92.4 g, 0.26 mol) in 88% yield and with an assay of 99.1% (HPLC).

12, 13, and 14: Side-Product Isolation. Approximately 1 L of plant 2-propanol recrystallization mother liquor was concentrated at the rotary evaporator to an oil, and a portion of this was separated on a silica gel column using ethyl acetate–hexane as eluent. From this chromatography **12**, **13**, and **14** were isolated as and subsequently identified.

12 (1-benzyl-4-methyl-3-(1R-phenethyl)-1,3-dihydroimidazol-2-one, $\text{C}_{19}\text{H}_{20}\text{N}_2\text{O}$ [292.38 g/mol]): ^1H NMR 1.62 (s, 3H), 1.80 (d, 3H, $J = 7.1$), 4.63 (s, 2H), 5.49 (q, 1H, $J = 7.1$), 5.61 (s, 1H), 7.1–7.5 (m, 10H). MS (CI–methane) 293 ($\text{M} + 1$, 100), 217 (12), 189 (48), 105 (43), 91 (36).

13 (1-benzyl-3-(1R-phenethyl)-1,4-dihydro-3H-thieno[3,4-d]imidazol-2,6-dione, $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_2\text{S}$ [350.44 g/mol]): ^1H NMR 1.71 (d, 3H, $J = 7.1$), 3.12 (d, 1H, $J = 17.4$), 3.71 (d, 1H, $J = 17.4$), 4.95 (s, 2H), 5.61 (q, 1H, $J = 7.1$), 7.27–7.40 (m, 8H), 7.49 (dd, 2H, $J = 8, 1.5$). MS (CI–methane) 351 (M^+ , 100), 247 (34), 105 (98), 91 (55).

14 (3,3a-dibenzyl-1-(1R-phenethyl)-tetrahydro-thieno[3,4-d]imidazol-2,4-dione, $\text{C}_{27}\text{H}_{26}\text{N}_2\text{O}_2\text{S}$ [442.58 g/mol]): ^1H NMR 1.52 (d, 3H, $J = 7.1$), 1.96 (dd, 1H, $J = 12.1, 5.4$), 2.30 (dd, 1H, $J = 12.1, 3.1$), 2.58 (d, 1H, $J = 13.4$), 3.25 (d, 1H, $J = 13.4$), 4.19 (dd, 1H, $J = 5.4, 3.1$), 4.65 (d, 1H, $J = 15.8$), 4.79 (d, 1H, $J = 15.8$), 5.32 (q, 1H, $J = 7.1$),

(17) The olefinic signal in ^1H NMR overlaps with the phenethyl methine proton, nonetheless the pattern of the signals is unchanged.

7.10–7.40 (*m*, 15H). IR (neat) 3100, 1704, 1500, 700. MS (CI-methane) 443(M^+ , 78), 414(46), 367(59), 104(70), 90(100).

Pentanoic Acid, 5-[Hexahydro-2-oxo-1-(1*R*-phenylethyl)-3-(phenylmethyl)-4*H*-thieno[3,4-*d*]-imidazol-4-ylidene]-[3*a*-*S*-[1(*S),3*a*- α ,6*a*- α (9):** In a dry 500-mL double-walled reactor with nitrogen inlet were added Mg turnings (29.9 g, 1.23 mol) and 356 g of tetrahydrofuran (THF). This suspension was warmed to 30–35 °C. To the suspension was then added by drops 1,2-dibromoethane (10.52 g, 0.06 mol) until the Grignard initiated. Thereafter, 1,4-dichlorobutane (74.7 g, 0.59 mol) was added dropwise to the mixture, while maintaining an internal temperature of 30–35 °C (ca. 30 min). The mixture was then maintained at 30–35 °C for an additional 3 h (becoming a thick gray suspension) before it was diluted with an additional 474 g of dry THF and transferred to a 2-L reactor. *N,N*-tetramethylenediamine (29.2 g, 0.25 mol) was then added, and the mixture was cooled to –25 to –30 °C. Thereafter, a previously prepared solution of **8** (99.7 g, 0.28 mol) in THF (722 g) was added, maintaining an inner temperature of –20 to –25 °C, typically completed within 1 h. After the addition was complete the reaction was stirred at –20 to –25 °C for an additional 1 h before gaseous CO₂ was passed over the surface of the mixture while maintaining –20 to –25 °C. The CO₂ addition was judged to be complete after all exothermic behavior ceased and the solution changed appearance to a light-gray suspension. The reaction mixture was then quenched into a previously prepared solution of 10% H₂SO₄ (aqueous, 1926 g). Toluene (1821 g) was then added, and the mixture was stirred for 30 min before the layers were separated. The organic phase was then concentrated to approximately 1340 g, 3.7 g of concentrated H₂SO₄ was added, and the mixture was warmed to 65 °C and held there for 1 h. The mixture was cooled to room temperature and washed 3× with 300 mL of water. The organic phase was then concentrated, and 216 g of ethyl acetate was added and the temperature stabilized at 60 °C. Heptane (475 g) was then added over about an hour. Normally, crystals would form at about half the heptane addition; if not, a few seed crystals were added. The precipitate was cooled to 0 °C and collected by filtration. After washing with a little heptane the off-white solid was dried overnight in a vacuum oven at 60 °C. Typical yield: 98.5 g; HPLC assay 95.8%, 78.4% overall yield.

22, 23, 24, and 15: Side-Product Isolation. From a normal **9** reaction carried out in the same fashion as described above (except that the reaction mixture after **8** addition was allowed to warm to room temperature for 1 h before being quenched into aqueous H₂SO₄ (no CO₂) and extracted into toluene) was obtained a mixture of products in which **22** was dominant. After the toluene was concentrated until a thick oil remained, silica gel column chromatography was carried out using ethyl acetate–hexane as eluent to obtain compound **22**.

22 (1-benzyl-5-(1-hydroxy-cyclopentyl-4-mercaptomethyl-3-(1*R*-phenethyl)-imidazolin-2-one, C₂₄H₃₀N₂O₂S [410.58 g/mol]): clear oil, ¹H NMR 1.39 (*t*, 1H, *J* = 8.3, SH, disappears slowly with D₂O), 1.55–1.77 (*m*, 8H), 1.80

(*d*, 3H, *J* = 7.1), 2.08 (*s*, 1H, OH disappears with D₂O), 2.58 (*dd*, 1H, *J* = 14.4, 7.8), 2.69 (*td*, 1H, *J* = 14.7, 9.2, 2.7), 3.52 (*d*, 1H, *J* = 7.4), 3.80 (*td*, 1H, *J* = 6.8, 2.9), 4.40 (*d*, 1H, *J* = 16.4), 4.80 (*d*, 1H, *J* = 16.4), 5.31 (*q*, 1H, *J* = 7.0), 7.10–7.55 (*m*, 10H); ¹³C NMR 17.4 (*q*), 24.3 (*t*), 24.9 (*t*), 33.8 (*t*), 36.9 (*t*), 37.5 (*t*), 46.7 (*t*), 50.8 (*d*), 59.7 (*d*), 65.4 (*d*), 67.1 (*s*), 127.3–128.7 (*m*), 136.8 (*s*), 141.9 (*s*), 160.7 (*s*); IR(neat) 3530*w*, 3410*m*, 1690*s*, 1500*m*, 1460*s*, 1400*m*.

A sample of **22** was dissolved in toluene and treated with a few drops of concentrated sulfuric acid and stirred at room temperature for several hours. TLC analysis showed little reaction; thus, an additional portion of sulfuric acid was added, and the reaction mixture was heated to 60–65 °C. Slow conversion commenced, but it was necessary to add three more portions of sulfuric acid and heat for another 11 h to get the reaction to complete. When the reaction was judged complete, it was cooled to room temperature and diluted with additional toluene, and the sulfuric acid was removed with water and saturated aqueous NaHCO₃ washes. The toluene solution was dried over Na₂SO₄, filtered, and concentrated. A portion of the oil that ensued was chromatographed over silica gel using ethyl acetate–hexanes as eluent to afford compound **23** as a clear oil.

23 (spirocyclic thioether, C₂₄H₂₈N₂OS [392.56]): ¹H NMR 1.58 (*d*, 1H, *J* = 6.8), 1.50–1.80 (*m*, 8H), 2.04 (*dd*, 1H, *J* = 12.7, 6.5), 2.32 (*dd*, 1H, *J* = 12.5, 7.1), 3.77 (*d*, 1H, *J* = 10.3), 3.84 (*d*, 1H, *J* = 15.5), 4.20 (*dt*, 1H, *J* = 10.5, 6.6), 5.12 (*d*, 1H, *J* = 15.5), 5.24 (*q*, 1H, *J* = 6.8), 7.10–7.40 (*m*, 10H); ¹³C NMR 15.1 (*q*), 24.3 (*t*), 24.9 (*t*), 33.8 (*t*), 36.9 (*t*), 37.5 (*t*), 46.7 (*t*), 50.8 (*d*), 59.7 (*d*), 65.4 (*d*), 67.1 (*s*), 127.3–128.7 (*m*), 136.8 (*s*), 141.9 (*s*), 160.7 (*s*); IR(neat) 1690*s*, 1652*m*, 1440*m*, 1420*m*.

A small sample of **22** was treated with I₂/KOH as described in the literature.¹³ After workup and column chromatography compound **24** could be isolated after silica gel column chromatography.

24 (dimeric disulfide, C₄₈H₅₉N₄O₄S₂ [820.14]): clear oil, ¹H NMR 1.50–1.78 (*m*, 16H), 1.85 (*d*, 6H, *J* = 7.1), 2.63 (*dd*, 2H, *J* = 14.1, 3.6), 3.02 (*dd*, 2H, *J* = 14.4, 7.8), 3.32 (*d*, 2H, *J* = 7.3), 3.78 (*td*, 2H, *J* = 7.3, 3.6), 4.22 (*d*, 2H, *J* = 16.0), 4.88 (*d*, 2H, *J* = 15.9), 5.00 (*q*, 2H, *J* = 7.3), 7.10–7.40 (*m*, 20H); ¹³C NMR 17.5 (*q*), 23.4 (*t*), 23.7 (*t*), 38.1 (*t*), 39.4 (*t*), 40.0 (*t*), 48.3 (*t*), 52.7 (*d*), 58.7 (*d*), 63.3 (*d*), 83.0 (*s*), 127.1–128.7 (*m*), 137.7 (*s*), 142.3 (*s*), 162.5 (*s*); IR (neat) 3390*m*, 1690*s*, 1460*m*, 1450*s*. MS (FAB) 823(4), 822 (10), 821 (26), 820 (57), 819 (100), 818 (10), 817 (15), 715 (50), 611 (60); calculated isotope pattern for C₄₈H₅₉N₄O₄S₂ 823 (3), 822(9), 821 (26), 820 (57), 819 (100).

As described in the text, a smaller-scale normal **9** reaction kept at –25 °C was quenched into DCI/D₂O before it could be reacted with CO₂. After extraction into toluene and reaction with concentrated H₂SO₄ (as described in the experimental details for **9**, above) and aqueous workup a toluene solution was obtained which contained **15(D)** mixture (mostly **25**). The toluene solution was concentrated, and **15-(D)** was isolated by silica gel column chromatography from the thick oil which remained. **15(D)** with a much lower

methyl deuterium incorporation could also be obtained after CO₂ introduction from the toluene mother liquor with a similar DCl/D₂O quench.

15(D), (C₂₄H₂₇DN₂OS [393.55]): oil, ¹H NMR 0.82 (*tt*, 2H, *J* = 7.3, 1.9), 1.29–1.34 (*m*, 2H), 1.60 (*d*, 3H, *J* = 7.1), 1.89–1.98 (*m*, 2H), 2.31 (*dd*, 1H, *J* = 12.2, 4.3), 2.43 (*dd*, 1H, *J* = 12.0, 5.7), 3.97 (*d*, 1H, *J* = 15.6), 4.15–4.24 (*m*, 2H), 4.90 (*d*, 1H, *J* = 15.7), 5.32 (*q*, 1H, *J* = 7.1), 5.38 (*t*, 1H, *J* = 7.1), 7.18–7.43 (*m*, 10H); ²D NMR (CCl₄, 76.8 MHz) 0.90 (broad *s*), 5.05 (broad *s*); ¹³C NMR 13.4 (*t* in fully ¹H decoupled ¹³C spectrum, CH₂D), 16.9 (*q*), 22.1 (*t*), 33.6 (*t*), 38.1 (*t*), 44.9 (*t*), 50.7 (*d*), 57.4 (*d*), 65.4 (*d*), 126.3 (*d*), 127.2–128.6 (*m*), 136.5 (*s*), 137.3 (*s*), 141.9 (*s*), 158.8 (*s*). IR(neat) 2930*m*, 1695*s*, 1440*m*.

By contrast the ¹H- and ¹³C NMR spectra of **15** quenched with H₂SO₄ are essentially identical to those of the deuterium-quenched samples in all peaks except that connected to the

terminal methyl group. There normal **15** shows the same pattern in ¹H NMR except that the integral amounts to three protons. In the off-resonance decoupled ¹³C NMR case the normally quenched **15** shows only a singlet at 13.4 ppm.

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