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Andrastins A−D, *Penicillium roqueforti* Metabolites Consistently Produced in Blue-Mold-Ripened Cheese

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This is the first finding of andrastins in blue cheese as well as any other sample type. Here, they were produced by the secondary starter culture *Penicillium roqueforti*. After purification by normal-phase chromatography followed by combined reverse-phase ion-exchange chromatography, the andrastins A−D were detected by liquid chromatography combined with UV and high-resolution mass spectrometry. In 23 representative samples of European blue cheeses, andrastin A was consistently found in quantities between 0.1 and 3.7 μg/g of cheese (median 2.4 μg/g). Assuming the same molar response factors as for andrastin A, the B, C, and D analogues were present in approximately 5-, 3-, and 5−20-fold lower amounts than andrastin A, respectively. The andrastins are protein farnesyltransferase inhibitors and are capable of inhibiting the efflux of anticancer drugs from multidrug-resistant cancer cells. Thus, their presence in common blue cheese suggests a potential for a positive or negative impact on human health.

KEYWORDS: Andrastin, *Penicillium roqueforti*, blue-mold cheese, SPE, LC−MS, Oasis MAX

INTRODUCTION

Penicillium roqueforti has been used as a secondary starter culture for the ripening of blue-mold cheeses, such as Gorgonzola, Danablue, Roquefort, Bleu de Bresse, Blue Stilton, and Edelpilzkäse for centuries. Because of the coevolution with lactic acid bacteria, *P. roqueforti* is resistant to lactic acid, acetic acid, carbon dioxide, and several other lactic acid bacterial metabolites (*1*−*3*).

There are many false reports on mycotoxin production from *P. roqueforti*. This is partly because in a broad sense it consists of three species: *P. roqueforti*, *P. carneum*, and *P. paneum* (*4*). These species have very similar physiological and phenotypic characteristics and are thus very difficult to identify. This is very important because they have very different profiles of metabolite and mycotoxins and thus different toxicological responses (*4*, *5*).

Only *P. roqueforti* sensu stricto is used in the production of blue cheeses, and the only real mycotoxin it produces is PR toxin and its precursors eremofortins A−E. All of these compounds, however, are not found in cheeses, where the related PR imine and PR amide are detected instead (*6*, *7*). Roquefortine C is consistently produced in blue cheeses (*8*) and has occasionally been described as a mycotoxin (*9*); however, there is no significant toxicological data in the literature to support this premise (*10*). The antibacterial and immunosuppressive metabolite, mycophenolic acid (*11*), has been found to occur naturally in some blue and Manchego cheeses (*12*−*14*). Reports of production of patulin are incorrect and are a result of misidentification with *P. carneum* and/or *P. paneum* (*4*, *5*), two species not used as starter cultures.

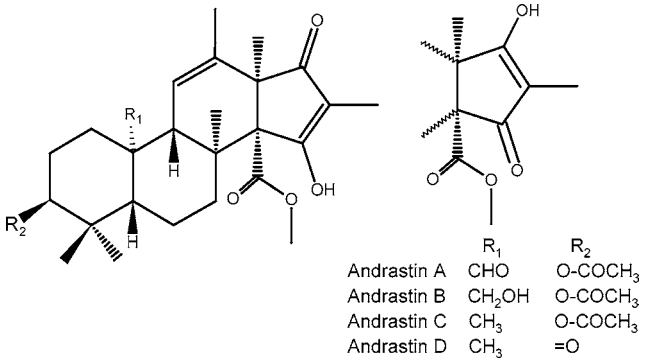

Figure 1. Structure of the andrastins. (Right) Tautomerism making them weak acids.

In addition to the previously described metabolites, we have for many years detected a major chromatographic peak, referred to as "Metabolite A" from all *P. roqueforti*, *P. carneum*, and *P. paneum* isolates as well as from numerous other penicillia (*4*, *5*). Recently, we identified this peak as the known compound andrastin A (*ent*-5α,14β-androstane) (**Figure 1**) by liquid chromatography (LC) combined with UV spectroscopy and high-resolution mass spectrometry (MS) (unpublished results).

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Table 1. Contents of Andrastin A and the Relative Peak Area of $[M - H]^-$ of Andrastins B, C, and D Compared to A

cheese	country[a]	A[b] (μg/g)	B[c] (% of A)	C[c] (% of A)	D[c] (% of A)
blue cheeses					
Blue, Castelleo	DK	2.7 ± 0.6[d]	25 ± 8	6 ± 2	4 ± 1
CremeBlue Klosterkrone,Høng	DK	1.2 ± 0.2	14 ± 3	23 ± 5	ND[e]
Danablue, St. Clemens, 60+	DK	2.4 ± 0.7	40 ± 18	ND	ND
Danablue, St. Clemens, 50+	DK	1.4 ± 1.1	9 ± 11	17 ± 20	7 ± 8
Black Castello	DK	3.7 ± 0.4	41 ± 6	113 ± 16	39 ± 6
Blue Castello (organic)	DK	3.5 ± 0.3	18 ± 2	78 ± 10	21 ± 3
Danablue, Rosenborg, Arla	DK	3.3 ± 1.0	26 ± 11	109 ± 47	12 ± 5
Danablue (organic),Thise	DK	3.4 ± 0.8	41 ± 15	123 ± 45	24 ± 9
Danablue, Gedsted (organic), Thise	DK	3.1 ± 0.2	25 ± 2	68 ± 5	10 ± 1
Danablue, 50+, Høng	DK	1.4 ± 0.5	11 ± 6	30 ± 18	5 ± 3
Bleu D'auvergne, 25% fat	Fr	2.9 ± 0.6	19 ± 6	38 ± 11	19 ± 6
Fourme d'Ambert	Fr	1.9 ± 0.2	21 ± 3	49 ± 8	ND
Saint Agur	Fr	3.7 ± 0.7	20 ± 5	84 ± 23	14 ± 4
Bresse Bleu, 55% fat	Fr	0.67 ± 0.2	6 ± 2	19 ± 7	6 ± 2
Gorgonzola, DOP Erborinato, Galbani	It	2.4 ± 0.3	24 ± 4	85 ± 15	18 ± 3
Gorgonzola 48+	It	1.4 ± 0.1	12 ± 1	78 ± 6	6 ± 1
Gorgonzola 48+, Igor	It	1.4 ± 0.2	17 ± 5	85 ± 22	11 ± 3
Magor, Gorgonzola, and Mascapone	It	0.08 ± 0.05[f]	ND	ND	ND
Gorgonzola, Erborinato	It	0.11 ± 0.08[f]	ND	ND	ND
Gorgonzola, Dolcelatte	It	2.5 ± 0.4	28 ± 6	108 ± 23	11 ± 2
Blue Stilton, 48+	UK	1.1 ± 0.1	5 ± 0	20 ± 2	3 ± 0
white cheeses					
White Castelleo	DK	ND	ND	ND	ND
Golden Castelleo	DK	ND	ND	ND	ND
Danish Farmbrie, Vejle Cheese	DK	ND	ND	ND	ND
French Farmbrie, light 11% fat	Fr	ND	ND	ND	ND

[a] DK, Denmark; Fr, France; It, Italy; and UK, United Kingdom. [b] Quantification based on LC–UV (262 ± 2 nm) from spiked white cheese, with MS confirmation. [c] Peak area of the $[M - H]^-$ ion compared to andrastin A $[M - H]^-$. [d] ± standard deviation (triplicate extractions). [e] ND = not detected. [f] Quantified by ESI–HR–MS because the peak was not detected by UV.

The identification was further validated by purifying andrastin A from *P. allii* by high-speed countercurrent chromatography and subsequent 1D and 2D NMR spectroscopy (unpublished results) matching the original data (*15*). In two ongoing projects on the chemotaxonomy of the *P. roqueforti* complex, we noticed that andrastins A−D were consistently produced on all laboratory substrates used as well as in grass and maize silage (unpublished results), leading to the hypothesis that they are also produced in blue cheese. Currently, there are no published studies showing if these metabolites are actually produced in any natural habitat. Andrastins are interesting anticancer drug candidates because they are potent farnesyltransferase inhibitors, especially of the RAS proteins, which are important for controlling cell division and the development of cancer (*16*−*19*). They can also enhance the accumulation of anticancer drugs in vincristine-resistant cancer cells (*16*). However, no data on their toxicological properties have been published except from their impact on human KB cells (*16*).

With andrastins showing such interesting biological activity, we decided to survey blue cheese for the presence of andrastins A−D. Thus, the analytical methodology was developed, and 21 blue cheeses, from Denmark, England, France, and Italy, were analyzed, using 4 Brie and Camembert cheeses as controls. The results may have important implications toward understanding the ecology of the ripening of blue cheeses and may also affect public health because of the anticarcinogenic properties of the andrastins.

MATERIALS AND METHODS

Andrastin A was purified from *Penicillium allii* and shown to be >95% pure by LC−UV and LC−MS. A stock solution was prepared in methanol (CH_3OH) (HPLC grade, Lab-Scan, Dublin, Ireland) and quantified by UV spectroscopy at 211 nm (pure methanol at ϵ 10 580) and 209 nm (in acidic CH_3OH at ϵ 9370) (*20*). UV absorption was measured in 10 mm quartz cuvettes (Hellma, Müllheim, Germany) on a Lambda 2 UV−vis spectrophotometer (Perkin−Elmer, Überlingen, Germany).

A total of 21 blue-mold-ripened cheeses (**Table 1**) were purchased in three local supermarkets and frozen at −22 °C until extraction. A total of 4 white-mold-ripened cheeses were also purchased and used as controls, because *P. camemberti* does not produce andrastins A−D (*5*). Samples from all cheeses were extracted within 14 days of freezing. Subsamples that were analyzed were taken from thin cross sections (1−2 mm) of the cheeses. These did not include crust, rinds, and other external nonedible part of the cheeses. Samples (0.3−0.6 g) were accurately weighed, mixed with approximately the same volume of silica gel 60 (0.015−0.040 mm) (Merck, Darmstad, Germany), and ground in a mortar. The mixture was transferred to an aluminum-foil wrapped 16-mL vial with 2 × 6 mL of dichloromethane (CH_2Cl_2) (HPLC grade, Lab-Scan), shaken for 2 h on a laboratory shaker at 100 rpm, and then kept at −18 °C for 2−5 days. The sample was concentrated in vacuo to approximately $^1/_3$ volume, after which 4 mL of hexane was added. The silica mix was transferred to a homemade solid-phase extraction (SPE) cartridge containing 1 cm^3 silica gel 60 tightly packed between 2 disks of 3 mm Vyon Sheet (porous high-density polyethylene) (FilterServe, West Midlands, U.K.) in a 5-mL disposable syringe. Prior to use, the cartridges were activated for 1 day at 110 °C and treated sequentially with 4 mL of CH_3OH, 4 mL of CH_2Cl_2, and 4 mL of hexane. The sample was applied, and excess solvent was drained; however, the SPE cartridge was not run dry. The cartridge was washed with 5 mL of CH_2Cl_2−hexane (HPLC grade, Sigma, Steinheim, Germany) (2:1) containing 1% formic acid (HCOOH) (analytical grade, Sigma), and the sample was eluted with 8 mL of CH_2Cl_2−CH_3OH (3:1), which was evaporated to dryness in vacuo. The solid residues were redissolved in 1 mL of H_2O−CH_3OH (6:4) and loaded onto a 60 mg Oasis MAX SPE mixed reversed-phase anion-exchange cartridge (Waters, Milford, MA), which had previously been conditioned with 2 mL of CH_3OH and 2 mL of H_2O−CH_3OH (6:4). Water was purified on a Milli-Q system (Millipore, Bedford, MA). After the sample was washed with 2 mL of CH_3OH, it was eluted with 3 mL pf H_2O−CH_3OH (1:9) containing 2% HCOOH, evaporated in

vacuo, redissolved in 500 μL of CH_3OH, and filtered through a 4 mm 0.45 μm PFTE syringe filter (Chromacol, Herts, U.K.).

Extraction efficiency was determined by spiking both white mold cheese (Danish Castello, white) and the pure silica with the andrastin A stock solution. The samples were allowed to dry for 1 h prior to extraction. The following amounts of andrastin A were added (from 30 or 100 μL of CH_3OH solution) to the samples: 11.1, 3.35, 1.11, 0.335, 0.111, 0.033, 0.0111, 0.00335, and 0.00111 μg.

Analysis of Andrastins. High-resolution LC−DAD−MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.), with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (*21*) and controlled by MassLynx 4.0 software. Negative ESI (ESI^-) was performed at a resolution >5000 (at half-peak height), with the $[M - H]^-$ ion of leucine enkephalin (*m*/*z* 554.2615) as the lock mass. Data were collected as centroid data from *m*/*z* 80 to 800. The capillary was held at 1800 V, and the potential difference between cone 1 and 2 was held at 80 V, to induce significant in-source fragmentation. The desolvation temperature was 450 °C, with the source kept at 120 °C. Separation of 3 μL of the sample was performed on a 50 × 2 mm ID, 3 μm, Luna C_{18} II column (Phenomenex, Torrance, CA) with a H_2O−acetonitrile (CH_3CN) gradient system. HCOOH, 200 μL/L, was added to the H_2O. The gradient was started at 40% CH_3CN (HPLC grade, Riedel-de Haën, Seelze, Germany) and increased linearly to 70% CH_3CN over 10 min, before raising to 100% over 1 min, and then holding at 100% for 4 min.

Data Analysis. The contents of andrastin A in the cheese samples were quantified from the UV trace at 262 ± 2 nm and confirmed from the reconstructed ion chromatograms of $[M - H]^-$: *m*/*z* 485.2540 and the fragments *m*/*z* 425.22 and 180.05. Andrastin B was detected from the *m*/*z* 487.2696 trace and confirmed by the fragments *m*/*z* 427.22 and 180.05. andrastin C was detected by *m*/*z* 471.2747 and confirmed by *m*/*z* 411.23 and 180.05. Andrastin D was detected by *m*/*z* 427.2485 and confirmed by the fragments *m*/*z* 353.20 and 180.05. For all MS traces, a window of ±0.02 Da was used.

RESULTS

Analysis, Extraction, and Sample Preparation. Initial experiments on sample preparation showed that the extremely high fat contents of the samples gave significant problems. Using CH_2Cl_2 extraction, after a two-time CH_3OH−hexane partitioning and then a reversed-phase SPE cleanup, gave poor reproducibility, presumably because it was not possible to dissolve the samples totally in 50% CH_3OH prior to SPE. An extraction using H_2O−CH_3OH−hexane (1:9:19, 1% HCOOH) followed by reversed-phase SPE cleanup worked better but instead gave problems with precipitation in the final extract. Therefore, the described method was chosen because it gave clean enough extracts for UV detection of andrastin A with a detection limit of ca. 50 ng (S/N 3) by UV and ca. 5 ng (S/N 5) by MS. The recovery from spiked cheese compared with spiked silica gel was determined by LC−UV to be 95 ± 4% ($n = 5$) and did not seem to be concentration-dependent.

Recovery experiments (results not shown) from the silica cleanup columns showed that the andrastins did not elute with CH_2Cl_2 alone but instead required CH_2Cl_2−CH_3OH (4:1) for elution. However, from the spiked cheese samples, the andrastins began to elute with CH_2Cl_2. It was therefore necessary to wash with CH_2Cl_2−hexane (1:1, 1% HCOOH) rather than pure CH_2Cl_2 to avoid the loss of analytes. Analysis of other *P. roqueforti* culture extracts and cheeses spiked with *P. roqueforti* extracts (results not shown) showed a recovery of 95−100% from the Oasis MAX columns.

Mass Spectrometry. The andrastins were remarkably stable under ESI^- conditions with no in-source fragmentation occurring, using 50 V between the cones (~80−100 V on other micromass instruments with Z-spray sources), and a cone voltage of 85 V was needed to get qualifier ion intensities greater than 50% of the $[M - H]^-$ ion. These conditions generally result in extensive fragmentation of most compounds rendering them undetectable.

Cheese Analyses. As seen in **Table 1**, all of the blue mold ripened cheeses contained andrastin A in quantities between 0.1 and 3.7 μg/g with a mean of 2.4 μg/g. If we assume the same molar response factors of the $[M - H]^-$ ions of all of the andrastins, the C analogue was present at $^1/_3$ the amount, the B analogue at $^1/_5$ the amount, and the D analogue at $^1/_{20}$−$^1/_5$ the amount. In the four white mold ripened cheeses used as controls, andrastins A−D were not detected. This was expected because they have never been detected in extracts from *P. camemberti* cultures (*5*). No correlation was observed between the level of sporulating mycelium (assessed visually) in the cheeses and the quantity of the andrastins (results not shown).

DISCUSSION

Quantitative extraction of small metabolites from cheese poses significant analytical problems because of the high quantities of fats and proteins in this matrix. Highly acidic aqueous CH_3OH solutions can be used for the extraction of alkaloids such as roquefortine C (*22*). However, for apolar compounds, enzymatic hydrolysis of the protein and/or fat, acidic hydrolysis of the protein, or alkaline hydrolysis of the fats (*23*, *24*) can be used depending on the stability on the target compounds. Grinding the cheese with sand (*25*) or Celite (*26*, *27*) can also be used to break the interactions with the matrix, as demonstrated here. Many analytical procedures for cheese utilize a liquid−liquid extraction defatting step using CH_3OH−hexane or a similar step. However, a similar approach was not possible in this case because of the rather apolar structure of the andrastins, which require 70−80% CH_3CN for elution from a C_{18} phase. The andrastins were retained under ion-exchange conditions on the Oasis MAX SPE cartridges because they exist as tautomers with two keto−enol forms (*18*), each with an acidic proton on the enol (**Figure 1**).

NMR studies of andrastin A showed that it is unstable in $CHCl_3$ when kept for longer periods, presumably because of the formation of hemiacetal(s) from the aldehyde and one of the alcohols. Photolysis of $CHCl_3$ with subsequent formation of HCl is also known (*28*). Therefore, further studies on andrastins should be performed using other solvents, such as ethyl acetate for extraction and adjusting the solvent polarity with hexane, toluene, or similar solvents prior to loading onto the silica SPE cartridge.

The lower slope obtained by MS of the spiked silica calibration curve (**Figure 2**) compared with the spiked cheese was a surprise because the opposite was expected. Our results thus indicate that the cheese samples contain interfering compounds, which enhance the ionization of andrastin A, presumably as a result of changing the surface tension of the droplets during ESI^-, which is known by some compounds (*29*).

The better standard deviation of the LC−UV quantification compared with the LC−ESI−MS (**Figure 2**) was expected because UV is not biased by changes in the ionization efficiency but is of course overall less sensitive and selective than ESI−HR−MS. The poorer selectivity of UV was, however, always complemented by ESI−HR−MS (**Figure 3**) to confirm the same peak shape and time delay between the detectors. Furthermore, the LCT mass spectrometer used in this study has a microchannel ion collector with a limited dynamic range, which can lead to saturation; therefore, even with the software dead-time

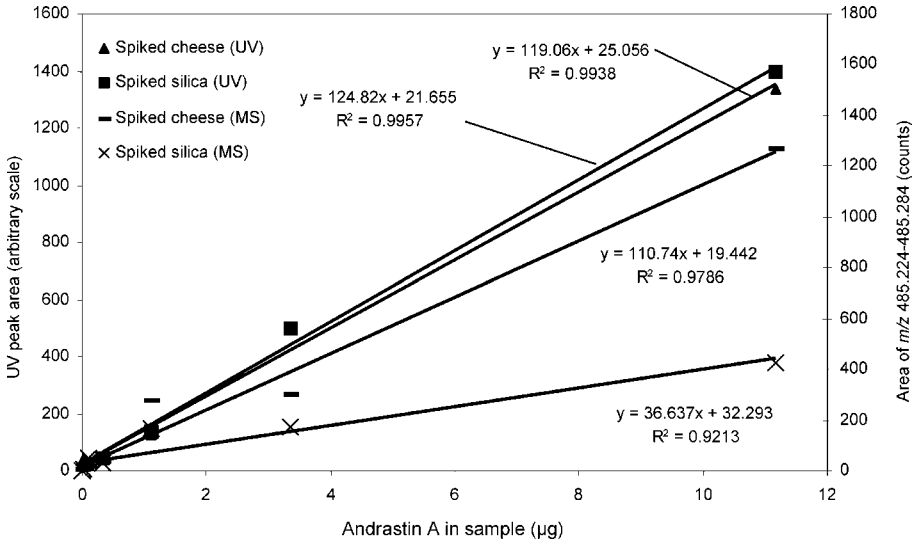

Figure 2. Calibration curves of andrastins A based on LC with UV detection (262 nm) and LC with negative electrospray ionization mass spectrometry.

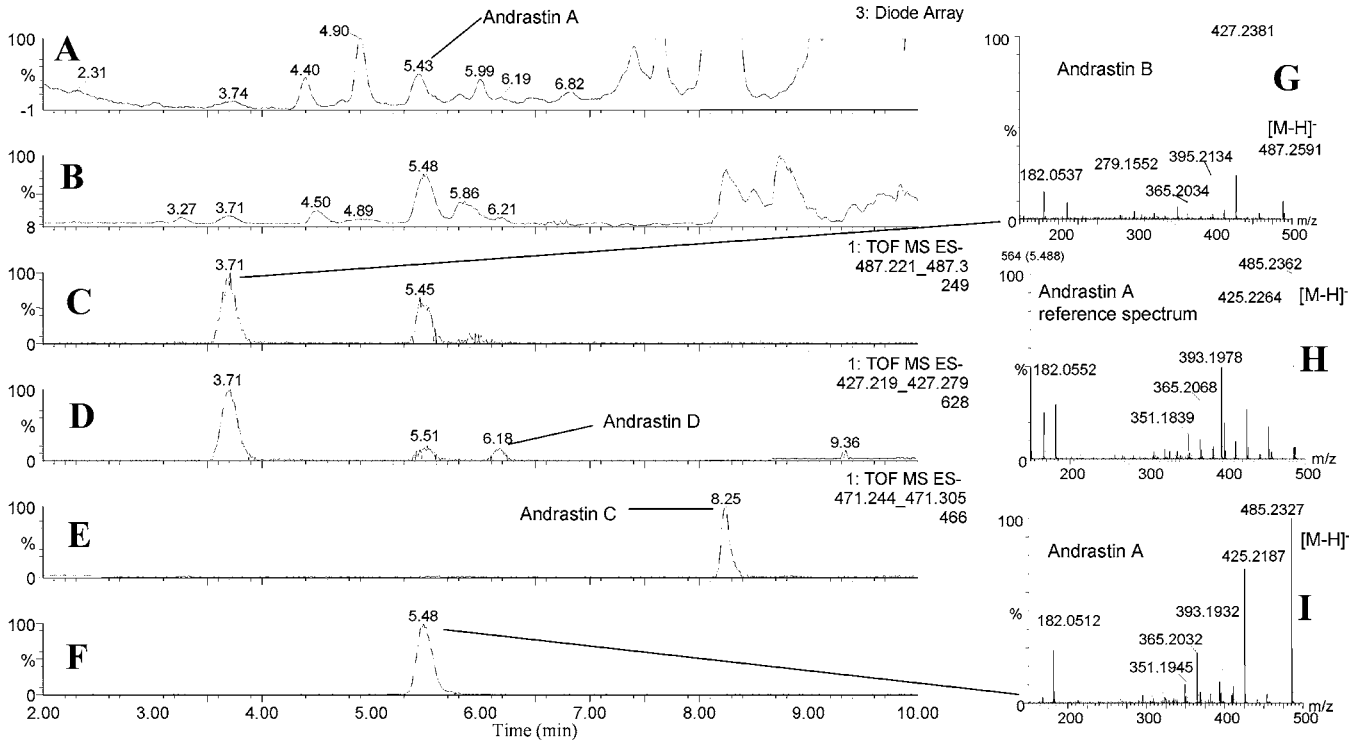

Figure 3. Chromatographic traces (not smoothed) of andrastins A–D in a Danish blue cheese. (A) UV signal (262 ± 2 nm, 0.05 min ahead of MS signals). (B) Total ion chromatogram. (C–F) Traces of $[M - H]^-$ of andrastins B, D, C, and A, respectively. (G) ESI^- peak apex spectra of andrastin B in the sample. (H) Andrastin A from the reference standard run. (I) Andrastin A from the sample.

correction, the linear range of the instrument is only between 2 and 3 decades (*30*, *31*), but at this point, the UV detector is capable of detecting the target compounds and the MS can still be used for confirmation.

The assumption that the andrastins have the same response factors is a very crude approximation because, e.g., the response factor for closely related type B-trichothecenes have been shown to vary by a factor of 5−6 (*32*). Then again, a much steeper gradient was used compared to this study, and because the response factor is very much dependent on the solvent composition, it is reasonable to assume a lower difference in response factors for the andrastins.

The andrastins were also detected in cheese with no visible sporulating mycelia, indicating that there is no correlation between sporulation and the concentration of these compounds as seen in the case of roquefortine C (*8*, *33*). When both the high amounts of andrastins produced on agar substrates (up to 10 times the amount of ergosterol) (unpublished results) and their relatively apolar character are taken into account, it suggests that these compounds have an intended role in this lipophilic environment or maybe even as a part of the fungal cell membrane. The latter hypothesis is supported by the lack of antibiotic activity against a very broad array of microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Bacteroides fragilis*, *Acholeplasma laidlawii*, *Pyricularia oryzae*, *Aspergillus niger*, *Mucor racemus*, *Candida albicans*, and *Saccharomyces sake* observed by Omura et al. (*20*), as well as *Trichophyton mentagrophytes* and *Cladosporium resinae* (unpublished results).

It is expected that the relative apolar structure of the andrastins facilitates crossing the cell membrane where they could directly affect RAS protein farnesyltransferase and *P*-phosphoglycoproteins as well as indirectly inhibit the efflux of the anticancer drugs (*16*−*18*).

In conclusion, we have developed the first published analytical method for andrastins, which were extracted from blue cheese by organic solvents, cleaned up by normal-phase SPE, combined by reversed-phase anion-exchange SPE, and detected by LC−UV−HR−MS. The study revealed that the andrastins are produced consistently by *P. roqueforti* during cheese ripening. Because the andrastins are farnesyltransferase inhibitors and active against cancer cells, the possible positive and negative effects of these compounds on human health need to be investigated further.

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